New antimicrobial anthraquinone 6,6'-bis (1,5,7-trihydroxy-3-hydroxymethylantraquinone) isolated from *Streptomyces* sp. isolate ERI-26

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**Abstract** The present report is about *Streptomyces* sp. isolate ERI-26 isolated from the soil sample of Nilgiri forest, Western Ghats. The methanol extract of ERI-26 showed good antimicrobial activity against tested microbes. The antimicrobial novel anthraquinones were purified by bioactivity-guided fractionation using a silica gel column and preparative HPLC. The compound was characterized and identified by UV, IR, NMR and MASS spectral data. The compound named as 6,6'-bis (1,5,7-trihydroxy-3-hydroxymethylantraquinone), showed significant antimicrobial activities against tested microbes. The isolated compound inhibited the tested bacterial growth, *Staphylococcus aureus* at 62.5 \(\mu\)g/ml, *Staphylococcus epidermidis* at 15.62 \(\mu\)g/ml, *Bacillus subtilis* at 62.5 \(\mu\)g/ml, fungi; *Trichophyton mentagrophytes* at 15.62 \(\mu\)g/ml *Trichophyton simii* at 15.62 \(\mu\)g/ml, *Aspergillus niger* at 7.81 \(\mu\)g/ml, *Aspergillus flavus* at 3.90 \(\mu\)g/ml, *Trichophyton rubrum* at 3.90 \(\mu\)g/ml, *Trichophyton rubrum 57/01* at 7.81 \(\mu\)g/ml, *Magnaporthes grisea* at 15.62 \(\mu\)g/ml and *Botrytis cinerea* at 3.90 \(\mu\)g/ml. Isolated anthraquinone compound and its antimicrobial activity were newly reported.

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1. Introduction

The emerging microbial infection and drug resistant pathogens are creating a major health problem throughout the world. In order to rectify these problems, searching for an effective, new antibiotic compound with significant drug mode of action is very important. The secondary metabolites from actinomycetes, particularly the members those belonging to the phylum Actinobacteria are having a wide variety of chemical
metabolites possessing strong biological activities (Spizek et al., 2010). Streptomyces is GC rich, Gram-positive, filamentous member of the phylum, Actinobacteria and it produces many pharmaceutically important secondary metabolites such as therapeutic enzymes, antibiotics, immunosuppressants, anti tumour agents and vitamins (Watve et al., 2001). Actinomycetes are usually Gram-positive bacteria containing a percentage of guanine, cytosine higher than 55%, and most of the actinomycetes produce mycelia. The actinomycetes are very interesting due to their ability to synthesize biologically active metabolites with diverse chemical molecules. (Valan Arasu et al., 2008). Streptomyces especially the genus Streptomyces were very potent producers of secondary metabolites including anthracyclines, aminoglycosides, macrolides, glycopeptides, nucleosides, peptides, polyenes, polyethers, and antetracyclines (Miyadoh, 1993). The widespread use of antibiotics in medicine plays a significant role in the emergence of resistant bacteria and specifically, misuse and/or overuse of antibiotics are the major causes (Mathew et al., 2007; Ferber, 2002; Goossens et al., 2005).

The actinomycete class produces novel secondary metabolites, and sometimes very unique metabolites which significantly show biological activities with low toxicity (Berdy, 2005; Kurtboke, 2012). Most of the antimicrobial compounds have been isolated and characterized from actinomycete group including anthracyclines, aminoglycosides, macrolides, glycopeptides, nucleosides, beta-lactams, peptides, polyketides, tetracyclines and actinomycins by Berdy (2005).

Number of antibiotics are isolated from extracellular of microbes which are diffused in culture media (Bode et al., 2002; Charoen Sopharat et al., 2008). The present studies are aimed to isolate the novel molecule from Streptomyces sp. ERI-26 and tested against pathogenic bacteria and fungi.

2. Materials and methods

2.1. Isolation and identification

The isolation and identification of selected Streptomyces isolate. ERI-26 have been reported in our earlier article (Valan Arasu et al., 2008).

2.2. Chemical

The pure organic solvents were used in this experiment. Hexane, ethyl acetate and methanol solvents were purchased from Ranbaxy laboratory, Saidapet, Chennai-15. The Silica gel was purchased from Merck (NJ, USA), which is used for thin layer chromatography (TLC) and column chromatography. All other chemicals were pure grade.

2.3. Extract preparation and isolation of active fraction by column chromatography

The isolate Streptomyces sp. isolate ERI-26 was grown in MNGA agar media and kept for incubation for 6 days. After the incubation period the culture with agar media was put into 500 ml of methanol in a conical flask. The content was allowed to diffuse the compounds in methanol. After that it was subjected to centrifuge (4000 rpm, 10 min) and extract was taken in methanol. The methanol phase was evaporated using vacuum rotary evaporator. Finally crude extract was obtained. The extract 5.25 g was subjected to fractionation using silica gel column chromatography. Totally 12 fractions were collected using the solvent system of chloroform, ethylacetate and methanol and their mixture. The active fraction 5 was taken for further isolation of active compound.

2.4. Reversed-phase high performance liquid chromatography (RP-HPLC) purification

The 5th fraction yielded 5 g. The fraction 5 was further purified with the help of preparative HPLC with an isocratic elution capability, ultraviolet spectrophotometer as detector and an auto sampler (Waters Alliance System). The purification of 5th fraction in preparative HPLC the solvent system were used: acetonitrile and aqueous acetic acid (15:85, v/v). The flow-rate was 3 ml/min and sample injection volume was 100 µl. The fraction was monitored on the screen also at 254 nm and the peak fraction was carefully collected. The fraction 5 was purified as two fractions. The second fraction B was taken for further structure elucidation which is shown in HPLC 99.14%.

2.5. Identification and characterization of anthraquinone by spectroscopic method

Fraction B was obtained from fraction 5 using preparative HPLC method. Fraction B was submitted to spectroscopic analysis. The 1H NMR (300 MHz), (AL-300 JEOL) spectra were measured and 13C NMR, AL-300 JEOL spectra were measured on a (75.45 MHz). The mass spectrum of the isolated compound (ESI-MS-JEOL instrument) was taken, IR spectrum of the isolated compound was taken from Shimadzu by KBr pellet method.

2.6. Tested microorganisms

The present study was antimicrobial screening of isolated compound B against following pathogenic microbes; Bacteria: Enterococcus faecalis ATCC 29212, Bacillus subtilis MTCC 441, Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, Staphylococcus epidermidis MTCC 3615, Klebsiella pneumoniae ATCC 15580, Pseudomonas aeruginosa ATCC 27853, Proteus vulgaris MTCC 1771. Fungi: Trichophyton rubrum MTCC 296, Trichophyton mentagrophytes 66/01, T. rubrum 57/01, Trichophyton simii 110/02, Aspergillus niger MTCC 1344, Epidermophyton floccosum 73/01, Aspergillus flavus, Curvularia lunata 46/0, Botrytis cinerea, Candida albicans MTCC 227 and Magnaporthe grisea. The tested microbes were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.7. Preparation of inoculums

The preparation of inoculums was followed by standard protocol. The bacterial cells were grown in Mueller Hinton Broth (Himedia) for 24 h at 37 °C. These cell suspensions were diluted with sterile MHB to provide initial cell counts of about

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10^4 CFU/ml. The fungal culture was grown in Sabouraud Dextrose Agar (SDA) slants in suitable temperature at 28 °C for 3 days. After incubation period the spores were collected from slants using sterile DD water and homogenized. The yeast inoculums were prepared by using Sabouraud Dextrose Broth (SDB) at 28 °C for 48 h.

2.8. Minimum inhibitory concentration

The minimum inhibitory concentration of the isolated compound tested against microbes using standard method for bacteria (Duraipandiyan and Ignacimuthu, 2009), fungi (CLSI, 2008) and yeasts (NCCLS, 2002). The isolated compound dissolved in suitable solvents. The concentration of the tested compound was 250 µg/ml. This was serially diluted twofold. The bacterial 100 µl of inoculums (10^3 CFU/ml) was inoculated in each well and 10^3 spore/ml of fungi, respectively. The Fluconazole for fungi and Ciprofloxacin for bacteria were included in the assays as positive controls. The plates were incubated in 24, 48 or 72 h at 27 °C for fungi, up to 9 days for dermatophytes. The bacterial plates were incubated for 24 h at 37 °C. After the incubation period the Minimum inhibitory concentration of the tested compound was observed and the lowest extract concentration was noted, showing no visible fungal growth after incubation time.

3. Results and discussion

3.1. Isolation and purification

Streptomyces isolate (ERI-26) methanol extract was subjected to chromatography separation. Totally 12 fractions were collected and identified by the screening method, it was reported in our previous article (Valan Arasu et al., 2008). The active fraction 5 was further separated and purified using semi-preparative HPLC. This fraction gave two fractions. Both the fractions purified and showed activity against tested microbes. The fraction A was already published (Duraipandiyan et al., 2014). The fraction B was presently reported. The pure compound was purified by using preparative HPLC. The purified compound was eluted at 5.94 min yielding 95 mg, purity was 99.14% in HPLC (Fig. 1).

3.2. Structure elucidation of compound 6,6′-bis (1,5,7-trihydroxy-3-hydroxymethylantraquinone)

Further elution of the column with the same solvent mixture gave compound which was crystallized as yellow orange crystals from aceton (yield 82 mg, mp.293 °C). The compound answered for phenol and also for quinone. TLC of the isolated compound showed single spot with ethyl acetate: methanol: formic acid 3:2:0.2 as developing system, (Rf = 0.5) turning positive ferric reaction for phenol and also answered for quinone. TLC of the isolated compound was 250 µg/ml. Fungi: T. rubrum 62.5 µg/ml, S. epidermidis at 15.62 µg/ml, B. subtilis 62.5 µg/ml. E. faecalis at 62.5 µg/ml and P. aeruginosa at 150 µg/ml. Fungi: T. mentagrophytes at 15.62 µg/ml. E. flocosum at 125 µg/ml, T. simii at 15.62 µg/ml, T. rubrum 296 at 62.5 µg/ml. T. rubrum 57 at 7.81 µg/ml, A. niger at 7.81 µg/ml, A. flavus at 3.90 µg/ml, M. cincerea 3.90 µg/ml and M. grisea at 15.62 µg/ml. (Table 1). The significant MIC values of tested anthraquinone skeleton showed against A. flavus and B. cincerea.

3.3. Antimicrobial activities of compound

The compound 6,6′-bis (1,5,7-trihydroxy-3-hydroxymethylantraquinone) was tested against bacteria and fungi. Minimum inhibitory concentration values for the compound are reported in Table 1. The compound (6,6′-bis (1,5,7-trihydroxy-3-hydroxymethylantraquinone) inhibited the growth of S. aureus at 62.5 µg/ml, S. epidermidis at 15.62 µg/ml, B. subtilis at 62.5 µg/ml, E. faecalis at 62.5 µg/ml and P. aeruginosa at 150 µg/ml. Fungi: T. mentagrophytes at 15.62 µg/ml, E. flocosum at 125 µg/ml, T. simii at 15.62 µg/ml, T. rubrum 296 at 62.5 µg/ml, T. rubrum 57 at 7.81 µg/ml, A. niger at 7.81 µg/ml, A. flavus at 3.90 µg/ml, M. cincerea 3.90 µg/ml and M. grisea at 15.62 µg/ml. (Table 1). The significant MIC values of tested anthraquinone skeleton showed against A. flavus and B. cincerea.
the plant, microbes and insect. It is interesting to observe however that recently many reports showed that plant anthraquinones have been reported from *Streptomycetes* (Cui et al., 2008). Many researchers have reported anthraquinones from actinomycetes. For instance, Cui et al. (2006) have reported that two anthraquinones (Aloesaponarin II and 1,6-dihydroxy-8-hydroxymethylanthraquinone) were isolated from marine Actinomycete isolate M097.

Two novel anthraquinones, were isolated from culture of *Micromonospora* sp. named as lupinacidins A (Ia) and B (Ib), are isolated and Lupinacidins show significant inhibitory effects on the invasion of murine colon 26-L5 carcinoma cells without inhibiting cell growth (Igarashi et al., 2007).

Increasing emergence of resistant pathogens emphasizes the need for new and effective antimicrobials. In this investigation, many actinomycete isolates are competent of producing antimicrobial compounds, active against various infectious diseases causing resistant bacteria (Yoo et al., 2007; Sohng et al., 2008; Mellouli et al., 2003). The isolated compound from *Streptomyces* sp. ERI 26 inhibited the growth of *P. aeruginosa* at 150 μg /ml. The same organism *P. aeruginosa* was inhibited by the *Streptomyces* sp.KH003 at 50 mg /ml of crude extracts. Seung Sik Cho et al., 2012, reported newly isolated *Streptomyces* sp. CS392 producing antimicrobial compounds which were active against pathogenic microbes.

Previously we have reported an anthraquinone 2,3-dihydroxy-9,10-anthraquinone isolated from *Streptomyces galbus* ERINLG-127 ethyl acetate extract which showed good antimicrobial activity against tested bacteria and fungi. The compound showed significant MIC values of 12.5 μg/mL against *P. aeruginosa*, *Salmonella typhimurium*, *K. pneumoniae* (ESBL-3894), *K. pneumoniae* (ESBL-3971), and *S. aureus* (MRSA) (Balachandran et al., 2014).

Poumale et al. (2006) reported that new anthraquinone isolated from marine *Streptomyces* sp. which is named as 8-hydroxy-3-methoxy-1-propylanthraquinone and 3,8-dihydroxy-1-propylanthraquinone and the compound showed activity against bacteria at a concentration of 40 μg/ml.

| Tested organisms      | Tested compounds standard | C    | Streptomycin |
|-----------------------|---------------------------|------|--------------|
| *Staphylococcus aureus* | 62.5                      | <0.78|
| *Staphylococcus epidermidis* | 15.62   | 6.25 |
| *Bacillus subtilis*    | 62.5                      | <0.78|
| *Pseudomonas aeruginosa* | 150   | 25   |
| *Escherichia coli*     | >250                      | 6.25 |
| *Klebsiella pneumoniae* | >250                      | <0.78|
| *Enterococcus faecalis* | 62.5                      | 6.25 |
| *Proteus vulgaris*     | >250                      | 25   |

**Table 1** Minimum inhibitory concentrations of compound (6,6'-bis (1,5,7-trihydroxy-3-hydroxymethylanthraquinone)) tested microbes.

**Figure 1** HPLC chromatogram of compound 6,6'-bis (1,5,7-trihydroxy-3-hydroxymethylanthraquinone).

**Figure 2** Structure of the isolated compound 6,6'-bis (1,5,7-trihydroxy-3-hydroxymethylanthraquinone).
4. Conclusion

The present report is about the isolated novel antimicrobial antraquinone from *Streptomyces* sp. ERI 26, compound 6,6'-bis (1,5,7-trihydroxy-3-hydroxymethylanthraquinone) showed good antimicrobial activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.sjbs.2016.02.008.

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