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ORIGINAL ARTICLE

New antimicrobial anthraquinone 6,6[1]-bis (1,5,7-trihydroxy-3-hydroxymethylanthraquinone) 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[1]-bis (1,5,7-trihydroxy-3-hydroxymethylanthraquinone), showed significant antimicrobial activities against tested microbes. The isolated compound inhibited the tested bacterial growth, *Staphylococcus aureus* at 62.5 μg/ml, *Staphylococcus epidermidis* at 15.62 μg/m, *Bacillus subtilis* at 62.5 μg/ml, fungi; *Trichophyton mentagrophytes* at 15.62 μg/m *Trichophyton simii* at 15.62 μg/ml, *Aspergillus niger* at. 7.81 μg/ml, *Aspergiller flavus* at 3.90 μg/ml, *Trichophyton rubrum* 296 at 62.5 μg/ml, *T. rubrum* 57/01 at 7.81 μg/ml, *Magnaporthe grisea* at 15.62 μg/ml. and *Botrytis cinerea* at 3.90 μg/ml. Isolated anthraquinone compound and its antimicrobial activity were newly reported.

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

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metabolites possessing strong biological activities (Spizek et al., 2010). *Streptomyces* is GC rich, Grampositive, 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 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). Streptomycetes especially the genus Streptomyces were very potent producers of secondary metabolites including antibacterial enzymes and toxins (Ren et al., 2010). Major types of antibiotics produced by Streptomyces are aminoglycosides, anthracyclines, glycopeptides, b-lactams, macrolides, 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; Kurtböke, 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;Charoensopharat 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 (RPHPLC) 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 ^{1}H NMR (300 MHz), (AL-300 JEOL) spectra were measured and ^{13}C 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 15380, *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

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 (10^8 CFU/ml) was inoculated in each well and 10^4 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[1]-bis (1,5,7-trihydroxy-3-hydroxymethylanthraquinone)

Further elution of the column with the same solvent mixture gave compound which was crystallized as yellow orange crystals from acetone (yield 82 mg, mp.293°). The compound answered for phenol and also for quinone. TLC of the isolated compound showed single spot with ethyl acetate: methanol: acetic acid 3:2:02 as developing system, (Rf = 0.5) turning pink on exposure to ammonia vapour. Its molecular formula is $C_{30}H_{18}O_{12}$.

UV λ_{max} MeOH nm: 222,246,272,316,439. IR γ_{max} KBR cm^{-1}: 3429 (hydroxyl), 2924, 2853, 1630 (chelated quinone carbonyl), 1609, 1570, 1450, 1386, 1363, 1421, 1257, 1224, 1100, 1082, 1063, 878, 761, 731. ^{1}H NMR (δ, DMSO-d_6, 300 MHz): 7.27 (2H, brs, H-2 and H-2′), 7.65 (2H,brs, H4 and H-4′), 7.32 (2H,brs, H-8 and H-8′), 12.22 and 12.76 (2H each, brs, 4× chelated OH), 11.86 (2H, brs, C-7OH and C-7′ OH) 4.60 (4H, brs, 2× C$\underline{H_2}$OH). ^{13}C NMR (δ, DMSO-d_6, 75 MHz): 159.52 (C-1 and C-1′) 120.89 (C-2 and C-2′), 153.30 (C3 and C-3′), 117.22 (C-4 and C-4′) 132.22 (C-4a and C-4′a), 161.45 (C-5 and C-5′), 112.86 (C-6 and C-6′), 160.07 (C-7 and C-7′), 109.42 (C-8 and C8′), 132.83 (C-8a and C-8′a), 189.86 (C-9 and C-9′), 114. 09 (C-9a and C-9′a), 181.01 (C-10 and C-10′), 107.77 (C-10a and C-10′a), 61.99 (2× CH_2OH). EI-MS (m/z): 551 $[M\text{-}H_2O\text{-}H]^+$. The above spectral data showed the compound B to be 6,6[1]-bis (1,5,7-tri hydroxy-3-hydroxymethylanthraquinone) which was novel (Fig. 2).

3.3. Antimicrobial activities of compound

The compound 6,6[1]-bis (1,5,7-trihydroxy-3-hydroxymethylan thraquinone) was tested against bacteria and fungi. Minimum inhibitory concentration values for the compound are reported in Table 1. The compound (6,6[1]-bis (1,5,7-trihydroxy-3-hydro xymethylanthraquinone) 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. floccosum* 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, *B. cinerea* 3.90 μg/ml and *M. grisea* at 15.62 μg/ml. (Table 1). The significant MIC values of tested anthraquinone showed against *A. flavus* and *B. cinerea*.

Above isolated compound spectral data showed anthraquinone skeleton. The spectral data closely resembled to that of citreorosein (ω-hydroxyl emodin) an anthraquinone isolated from plants and fungi (Fujimoto et al., 2004). Compound was isolated as yellow orange crystals from acetone (m.p. 293°); it showed the molecular formula as $C_{30}H_{18}O_{12}$ based on mass, ^{1}H and ^{13}C NMR (DEPT) spectral data. It gave positive ferric reaction for phenol and also answered for quinone by giving bluish pink colour with alcoholic NaOH. The UV spectrum showed maxima at 222,246,272,316 and 439. The IR spectrum similarly showed the presence of hydroxyl (3429 cm^{-1}) and only chelated quinone carbonyl (1630 cm^{-1}). The small peak corresponding to unchelated quinone carbonyl which appears around 1664 cm^{-1} in anthraquinones (Thomason, 1977) was absent.

Compound was shown to be dimer of compound (Duraipandiyan et al., 2014), linked at C-6 by its ^{1}H and ^{13}C NMR spectra. The two meta linked aromatic proton H-2 and H-4 adjacent to the hydroxyl methyl group appeared as unresolved broad singlet as usual at δ 7.27 and 7.65. H-8 appeared as singlet δ 7.32. The methylene proton of the hydroxyl methyl group at C-3 appeared as broad singlet at δ 4.60. The chelated hydroxyl groups appeared at δ 12.22 and 12.76. The unchelated hydroxyl groups at C-7 appeared at δ 11.86. In the ^{13}C NMR spectrum the linkage of C-6 and C-6[1] in the dimeric structure of isolated compound is evident since C-6 appeared as a singlet in compound and moved down field to δ 112.86 from δ 107.88. It is also found that C-5 and C-7 undergo a slight upfield shift.

Isolated compound 6,6[1]-bis (1,5,7-trihydroxy-3-hydroxyme thylanthraquinone) is new anthraquinone hitherto unreported from fungi, insects, plants and microbes. The oxynation pattern of the compound does not relate to anthraquinones from

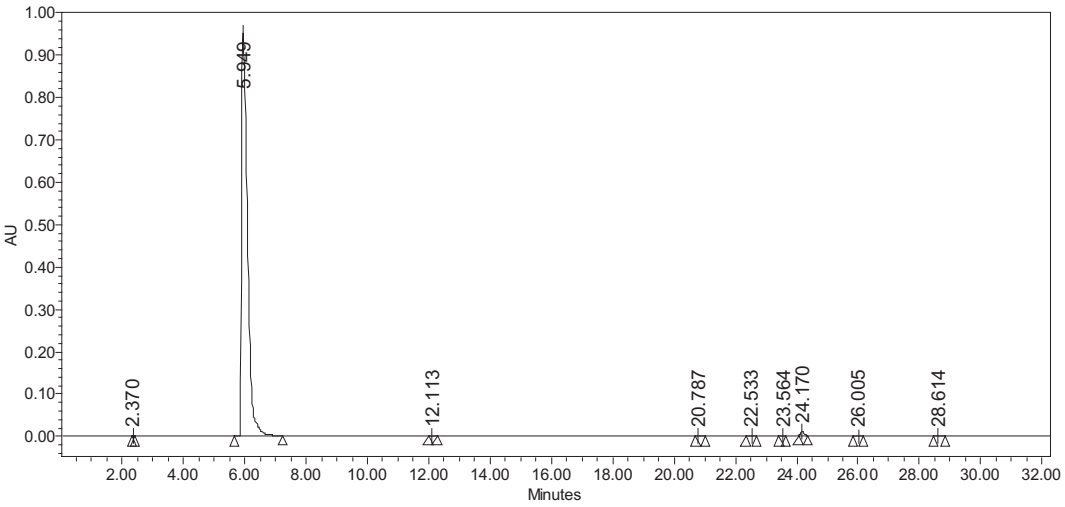

Figure 1 HPLC chromatogram of compound 6,6[1]-bis (1,5,7-trihydroxy-3-hydroxymethylanthraquinone).

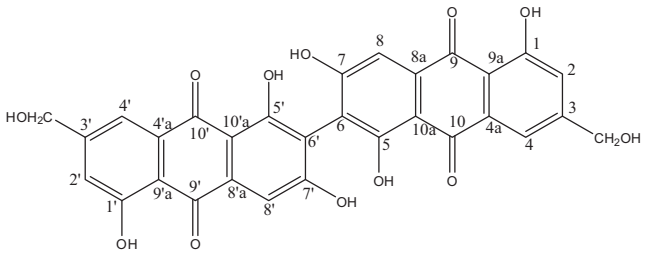

Figure 2 Structure of the isolated compound 6,6[1]-bis (1,5,7-trihydroxy-3-hydroxylmethylanthraquinone).

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-dihy droxy-8-hydroxymethylanthraquinone) were isolated from marine Actinomycete isolate M097.

Two novel anthraquinones, were isolated from culture of *Micromonospora* sp. named a 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

Table 1 Minimum inhibitory concentrations of compound (6,6[1]-bis (1,5,7-trihydroxy-3-hydroxylmethylanthraquinone) tested microbes.

Tested organisms	Tested compounds standard	
Bacteria	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
Fungi	Fluconazole	
Trichophyton mentagrophytes	15.62	25
Epidermophyton floccosum	125	12.5
Trichophyton rubrum 296	62.5	< 12.5
Trichophyton rubrum 57/01	7.81	25
Trichophyton simii	15.62	< 12.5
Curvularia lunata	> 250	< 12.5
Aspergiller flavus	3.90	25
Magnaporthe grisea	15.62	> 100
Botrytis cinerea	3.90	25
Aspergillus niger	7.81	100
Candida albicans	125	> 100

Compound: 6,6[1]-bis (1,5,7-trihydroxy-3-hydroxylmethylanthraquinone). Standard: Streptomycin (antibacterial agent), Fluconazole (antifungal agent).

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-hyd roxy-3-methoxy-1-propylanthraquinone and 3,8-dihydroxy-1-propylanthraquinone and the compound showed activity against bacteria at a concentration of 40 µg/ml.

4. Conclusion

The present report is about the isolated novel antimicrobial anthraquinone from *Streptomyces* sp. ERI 26, compound 6,6[1]-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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