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Fermentation, Isolation of Mithramycin from *Streptomyces* of Playa Region and its Novel Anti-MRSA and Anti-VRE Activity

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Abstract

A *Streptomyces* sp. PM1129877 was isolated from the playa region of the state of Rajasthan, India. By bioactivity guided isolation Mithramycin (Plicamycin), a reported antitumor compound was isolated, purified and characterized from the fermented broth of the microbial strain. Antibacterial and Antifungal studies revealed that mithramycin exhibited potent antibacterial properties. It displayed a minimum inhibitory concentration (MIC) range of 0.125-0.25 µg/ml against methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) strains with MIC_{90} of 0.25 µg/ml. Against vancomycin resistant enterococci (VRE) and vancomycin sensitive enterococci (VSE) strains, it displayed an MIC range of 1-16 µg/ml with MIC_{90} of 2.0 µg/ml. This is the first report of anti-MRSA and anti-VRE activity by mithramycin. This new use of mithramycin activity can boost the hope for therapeutics against various unmet medical needs associated with these resistant strains.

Keywords: Antibacterial; Mithramycin; MRSA, *Streptomyces;* VRE

Introduction

Mithramycin (Plicamycin, Mithracin, MIT) is a bright yellow, crystalline solid belonging to a group of aureolic acid type of antibiotics; produced naturally by a variety of *Streptomyces* sp. especially *S. argillaceus*, *S. plicatus* and *S. tanashiensis* [1-3]. Structurally, it is an aromatic polyketide antibiotic containing aglycon moiety, derived from the series of condensation reactions, catalyzed by a Type II polyketide synthase [4,5]. It is a known antitumor agent and acts on variety of tumor cell lines [6-8]. Antitumor properties of mithramycin are attributed to its role in inhibition of DNA dependent RNA synthesis. In the presence of Mg^{++}, it cross-links to G+C rich regions of DNA, thereby preventing the binding of Sp (specificity proteins) transcription factors, consequently affecting transcription [9-12].

Although majority of work related to mithramycin is concerned with its antitumor properties, it is also known to exhibit activity against Gram positive bacteria [13,14]. However, the organisms, *Staphylococcus aureus* or *Bacillus subtilis*, have merely been used as a tool in bioassay guided purification of mithramycin [2].

Globally, in last two decades, prevalence of the antibiotic resistance among the MRSA, penicillin-resistant *Streptococcus pneumoniae* (PRSP) and vancomycin resistant enterococci (VRE) has been observed against variety of antibiotics [15-19]. Moreover, incidences of resistance of Gram positive bacterial infections against the new drugs such as Linezolid and Daptomycin have also been published [20,21]. To combat such infections, discovery of novel drugs and/or novel scaffolds is of utmost importance. However new drug discovery is a long term and uncertain process with high expenditure. Another alternative to tackle such problem could be repositioning of the existing bioactive compounds in which known drugs are screened to establish their new bioactivity of desired interest [22]. For example, Rapamycin, a known immunosuppressant drug had been reported to exhibit anticancer activity against primary chronic lymphocytic leukemia cells [23]. Recently new anti-amyloidogenic activity was reported from previously known antibacterial antibiotic tetracycline [24]. Moreover mithramycin as a new candidate for developing new therapeutic drugs for neurodegenerative diseases has also been proposed [25]. Mithramycin has its beneficial effect for not only neurodegenerative diseases, but also a host of others, like cartilage degeneration in osteoarthritis, ER stress mediated apoptosis in hippocampus, erythroid differentiation and fetal haemoglobin production in thalassemia and sickle cell anaemia [26-28]

In the current study, we had isolated, purified and characterized mithramycin from a *Streptomyces* sp. PM1129877 isolated from a playa region of Thar Desert in Rajasthan, India. This playa region was hardly explored for isolation of actinomycetes producing bioactive compounds. During screening, we observed that mithramycin exhibited potent *in-vitro* antibacterial activity against variety of Gram positive pathogenic strains of MRSA and VRE. We confirmed these observations by *In vitro* testing of the compound against variety of Gram positive and Gram negative

bacteria including resistant strains obtained from clinics and hospitals. This is the first report on the novel activity of mithramycin against the MRSA and VRE strains.

Materials and Methods

The media ingredients such as Beef extract, Malt extract, Peptone, Yeast extract, Corn steep liquor etc. used for cultivation and fermentation of microbes were obtained from Hi Media Laboratories Pvt. Ltd, India. Salts such as NaCl, $CaCO_3$ $NaNO_3$ etc were procured from Merck Specialties Pvt. Ltd, India.

The normal phase flash chromatography was performed on CombiFlash R_f (Teledyne isco) using Redicep R_f 20 g silica column. HPLC analysis and UV spectra were monitored on Shimadzu LC-2010- PDA instrument (Shimadzu, Kyoto, Japan). The data were processed with LC-solution software (Shimadzu, Kyoto, Japan). Distilled LR grade solvents were used for column chromatography and TLC, HPLC grade solvents from Merck were used for preparative and analytical HPLC. TLC Silica gel 60 F_{254} plates were from Merck Specialties Pvt. Ltd, India. The NMR spectra were recorded on Bruker Avance at 500 MHz.

Sample collection, isolation and fermentation of the organism

Soil samples were collected from playa region of Pokhran (26.92°N 71.92°E), a remote region surrounded by rocky, sandy hills and salt lakes, located in Jaisalmer district in Thar Desert of Rajasthan state in India. The playa is a flat barren land in desert like areas with few shrubs and salt depositions seen intermittently. Pokhran playa is approximately 12 sq Km area of semi-arid loose sandy soil characterized with high concentrations of halite and traces of calcite, gypsum, proto-dolomite and anhydrite as a result of low rainfall and high evaporation to precipitation rate. The genetic identity and novelty of microbial extremophiles (i.e., bacteria, archaea, and protists) of this area remains largely unexplored [29]. The surface soil samples were collected with a sterile spatula and stored in sterile polythene containers. These samples were stored at ambient temperature and processed for microbial isolation at research centre of Piramal Enterprises Ltd., Mumbai, India.

Approximately 0.5 g of the collected sandy soil was suspended in 5 ml sterile saline (8.5 g/l NaCl in demineralised water) and vortexed for 1 min. This was diluted serially 1:10 up to 10^{-3}. 200 µl of 10^{-3} dilution of the soil suspension was surface spread on modified Bennet's agar medium containing (g/l), Glucose 10, Casamino acids 2, Yeast Extract 2, Beef Extract 2, Agar powder 15, final pH 7.2-7.5, supplemented with 50 g/l NaCl. The plates were incubated at 25 ± 1°C for 2-3 weeks and observed regularly for appearance of actinomycetes colonies. The producer strain of the mithramycin was one such isolate. The isolate was picked up, purified and maintained on modified ISP2 medium slants containing (g/l), Glucose 40, Yeast extract 40, Malt extract 100, Agar powder 15, final pH 7.5, supplemented with 50 g/l NaCl. The isolate was designated with code PM1129877.

A loopful of growth from 15 days old slant of this strain PM1129877 was inoculated in 274 (1) seed medium containing (g/l), Glucose 15, Peptone 7.5, Yeast extract 7.5, Corn steep liquor 5, NaCl 5, $CaCO_3$ 2, pH was adjusted to 7.5. The culture was incubated on shaker at 200 rpm for 72 h at 30°C. 4% (v/v) of seed inoculum was added in 20 L production medium 1M containing (g/l), Glycerol 30, Glucose 3, Peptone 3, Yeast extract 2, $CaCO_3$ 3, $NaNO_3$ 1, NaCl 30, final pH 7.5. The medium was supplemented with 15 g/l additional NaCl. The 200 production medium flasks (each containing 100 ml medium in 1 L capacity flasks) were incubated on shaker at 200 rpm for 96 h at 30°C. After incubation, contents of the flasks were pooled together and representative sample (100 ml) of this whole broth was extracted with equal volume of methanol under shaking condition for 1.5 h at 30°C. The contents were centrifuged and supernatant (Methanolic extract of whole broth, as mentioned in Table 1) was screened against Gram positive and Gram negative bacterial test cultures and yeasts and fungal test cultures. Antimicrobial activity was determined by whole cell agar well diffusion bioassay by boring 6 mm diameter wells in agarified medium and adding 50 µl samples to be tested in it [30]. Vancomycin (20 µg/ml), Gentamicin (50 µg/ml) and Amphotericin B (20µg/ml) were used as standard antibiotics for Gram positive bacteria, Gram negative bacteria and fungi respectively. Absolute methanol was used as solvent control.

Isolation, purification and characterization of the active compound

20 L of whole fermented broth was stirred with equal volume of absolute methanol for 1.5 h and the filtrate was concentrated to its half the volume under reduced pressure at 45°C. The concentrate was subjected to HP-20 resin adsorption chromatography and stirred for 1.5 h. Then HP-20 resin was washed with de-mineralised (DM) water and eluted with 80% aqueous methanol followed by absolute methanol. Both methanolic eluates were pooled and concentrated under reduced pressure at 40°C to obtain crude extract. The extract was subjected to normal phase (Silica gel, 100-200 mesh) flash column chromatography and the compounds were eluted by increasing proportion of ethyl acetate in petroleum ether. The fractions were pooled based on the similar silica TLC pattern (Mobile phase; Methanol: $CHCl_3$ = 1:9) observed at UV 254 nm. The bioactivity data indicated that active components were eluted by 20% ethyl acetate in petroleum ether. The final purification up to 99.5% was achieved by preparative HPLC. The pure compound was analyzed by analytical HPLC {(Column: LiChrospher, RP-18, 125 X 4.6mm, 5µm); Mobile phase: water and acetonitrile, 98/0, 0/15, 0/20, 98/21, 98/25 (% water/time in min)}. The compound was analyzed by LC-ESI MS on Micro QTOF of Bruker Daltonics. DMDO-d6 was used as solvent for these experiments and chemical shifts were referenced to the solvent peak at 2.50 ppm. The UV spectrum was obtained from a HPLC-photodiode array analysis of the compound using 15% acetonitrile/water [31].

Determination of minimum inhibitory concentration (MIC) values

The potency of purified mithramycin was determined by generating MIC values by the NCCLS (CLSI) macro-broth dilution

Table 1 : Antimicrobial Activity of Fermented Broth of *Streptomyces Sp*. PM1129877.

(zone of inhibition around the well in mm, well diameter 6 mm)

Details of samples used in whole cell agar well diffusion assay	***S. aureus* 209P, MSSA**	***S. aureus* ATCC 33591, MRSA**	***E. faecium* R-2-323, VRE**	***E. faecalis* ATCC 51575, VRE**	***E. coli* ATCC20732**	***C. albicas* HMR**	***C. krusei* GO3, Fluc^R**	***C. glabrata* HO5, Fluc^R**	***A. fumigatus,* HMR**
Methanolic whole broth of the organism	29	28	25	22	-	9vh	9h	-	12h
Vancomycin (20 µg/ml)	15	14	14	9h	NT	NT	NT	NT	NT
Gentamicin (50 µg/ml)	NT	NT	NT	NT	15	NT	NT	NT	NT
Amphotericin B (20 µg/ml)	NT	NT	NT	NT	NT	18	16	17	19
Methanol	-	-	-	-	-	-	-	-	-

NT: Not tested
Fluc^R: Fluconazole resistant
vh: very hazy zone of inhibition
h: hazy zone of inhibition
-: No zone of inhibition

method for aerobic bacteria M7-A5 [32]. Linezolid was used as a standard compound as it is currently used in clinic as therapeutic agent for MRSA infections. Mithramycin was suitably diluted in the concentration range of 0.0078-16 µg/ml. The concentration range for Linezolid used was 0.019-16 µg/ml. Absolute methanol was used as a solvent for making stock solutions of test compounds. Muller Hinton broth with two fold serial dilution of the compound was inoculated with 10^5 colony forming units/ml of test culture and incubated at 37°C for 24 hr. The visible growth (turbidity visible to naked eyes) was noted from each tube and the MIC values were recorded. The MIC of mithramycin was determined against 45 test organisms which included 37 Gram positive and 8 Gram negative clinical, in house and standard American Type Culture Collection (ATCC) strains.

Test organisms for bioactivity estimation

The bacterial test strains included Gram positive strains (total 37) obtained from in house strain bank of Hoechst Marion Roussel Ltd, India viz. *S. aureus* 209P MSSA, *S.aureus* E710 MRSA, *Enterococcus faecium* R-2-323 VRE (VanA), *E. faecium* R-2-322 VSE, *E. faecalis*-321 VSE, *Bacillus subtilis*-122, *B. megaterium* FH 1127, *B. licheniformis* ATCC 21552; standard strains procured from the American Type Culture Collection (ATCC), Manassas, USA included, *S. aureus* ATCC 33591 MRSA, *E. faecium* ATCC 51559 [Multidrug-resistant (ampicillin, ciprofloxacin, gentamicin, rifampin, teicoplanin, and vancomycin; vanA$^+$)], *E. faecalis* ATCC 51575 VRE, *E. faecalis* ATCC BAA 472 VRE, *E. faecalis* ATCC 51299 VRE (VanB) and *B. subtilis* ATCC 6633. 13 MRSA and 10 VRE strains were procured from private and government clinics/ hospitals in Mumbai–India.

Gram negative strains (total 8) obtained from in house strain bank of Hoechst Marion Roussel Ltd. India, included *Escherichia coli* ATCC 20732, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* M-35; standard strains procured from the American Type Culture Collection (ATCC), Manassas, USA included, *P. aeruginosa* ATCC 27853, *P. aeruginosa* ATCC BAA 47, *Acinetobacter baumannii* ATCC 19606 and *A.baumannii* ATCC BAA 747.

The fungal test strains used in agar well diffusion assay of fermented broth included *Candida albicans* HMR, *Candida krusei* GO3 Fluc^R, *Candida glabrata* HO5 Fluc^R and *Aspergillus fumigatus* HMR. These test strains were obtained from in house strain bank of Hoechst Marion Roussel Ltd, India.

Results and Discussion

The organism and its bioactivity

The colony of the producer strain PM1129877; which was picked after 2-3 weeks of incubation was leathery and surrounded by halo of translucent mycelia. It was circular to ovoid, 3-5 mm in diameter, with shining rough surface, convex with pointed tip, having yellow ochre substrate mycelia but devoid of aerial mycelia, sporulation and diffused pigments. On the basis of its morphological features, the organism was identified to be belonging to the genus *Streptomyces*. The culture (PM1129877) was deposited in Microbial Culture Collection [International Depositary Authority (IDA) at National Centre for Cell Science], Department of Biotechnology, Government of India] Pune, Maharashtra 411021- India.

The sample generated from methanolic whole broth extraction of PM1129877, had yellow color and exhibited exclusive and potent activity against Gram positive test cultures with very slight or no activity against yeasts/fungal and Gram negative test cultures (Table 1). For bioassay guided isolation of the compound, only Gram positive bacterial test cultures were selected.

Structural determination

The molecular weight of the compound m/z 1084.5 corresponded to the molecular formula $C_{52}H_{76}O_{24}$ of mithramycin (Table 2). The isolated compound (Figure 1 to Figure 4) was confirmed as mithramycin by different spectroscopic analysis

Table 2: Physical and Chemical Properties of Mithramycin.	
Appearance	**Yellow solid**
Molecular formula	$C_{52}H_{76}O_{24}$
Molecular weight	1084.5
LC-MS (m/z)	1083.5 $[M+H]^-$
UV λ_{max} Neutral nm	229,272,317 and 412
Solubility	Methanol, DMSO
Melting point	180-184°C

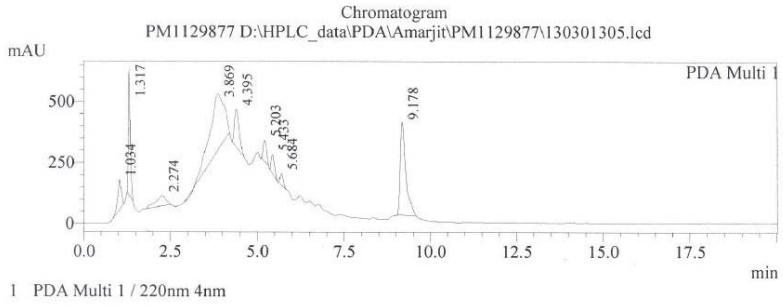

Figure 1: HPLC analysis of concentrate of methanolic eluate of HP20 resin (Arrow indicates Mithramycin peak).

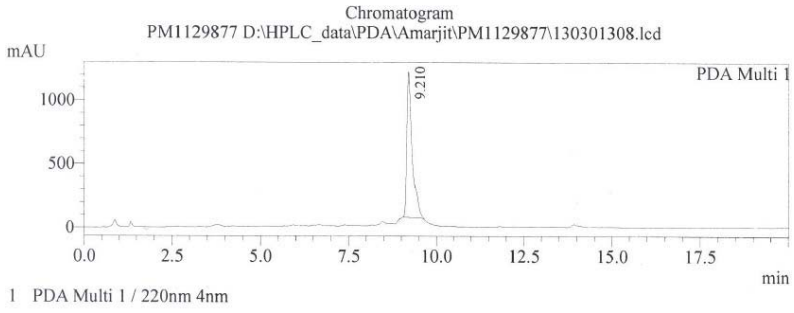

Figure 2: HPLC analysis of pure Mithramycin.

(Table 3 and Figure 3) such as LC-MS and ^1H NMR data [31,33,34].

Determination of potency of mithramycin by MIC estimation

Mithramycin exhibited potent growth inhibitory activity against Gram positive bacterial strains (Table 4), especially against clinical, resistant strains of MRSA and VRE. It inhibited the growth of MSSA and MRSA strains of *S. aureus* in the MIC range of 0.125-0.25 µg/ml with MIC_{90} value of 0.25 µg/ml. It also displayed activity against vancomycin resistant and vancomycin sensitive strains of *Enterococci* exhibiting MIC range of1-16 µg/ml with MIC_{90} value of 2 µg/ml. Clinical strains of *Enterococci* were inhibited in the MIC range of 1-2 µg/ml with MIC_{90} value of 2 µg/ml. *Bacillus* spp. were also strongly inhibited with MIC range of 0.031-1 µg/ml.

Apart from MIC estimation, mithramycin was also subjected to agar well diffusion assay against 8 Gram negative strains (Table 4) up to 256 µg/ml concentration. There was no zone of inhibition around any agar well for any of these test cultures, indicating mithramycin was ineffective against Gram negative test organisms. These results indicated that perhaps mithramycin was unable to penetrate the outer cell membrane barrier of the Gram negative test organisms and hence exhibited specific *in vitro* activity against different Gram positive test organisms only.

We started the present study with a hope to find a new antimicrobial compound, from microorganism, isolated from soil collected from untapped natural habitat, such as from playa region. In this journey, although we discovered a known molecule, mithramycin, it exhibited novel bioactivity against MRSA and VRE strains. Mithramycin with brand name Mithracin® was available in the market and was also FDA approved for the treatment of testicular cancer and for the treatment of hypercalcemia [35]. Despite hepatotoxicity and nephrotoxicity problems and other side effects, recently there

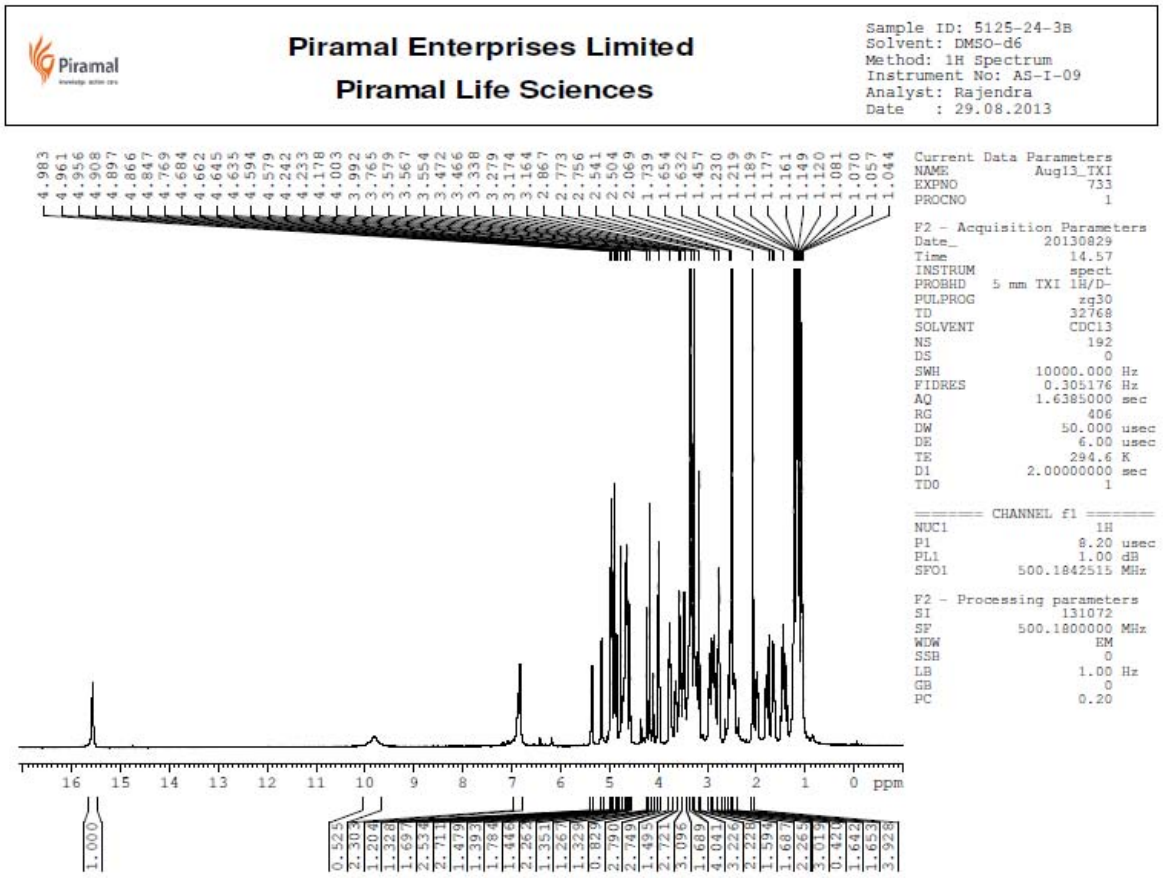

Figure 3: H^+ NMR of Mithramycin.

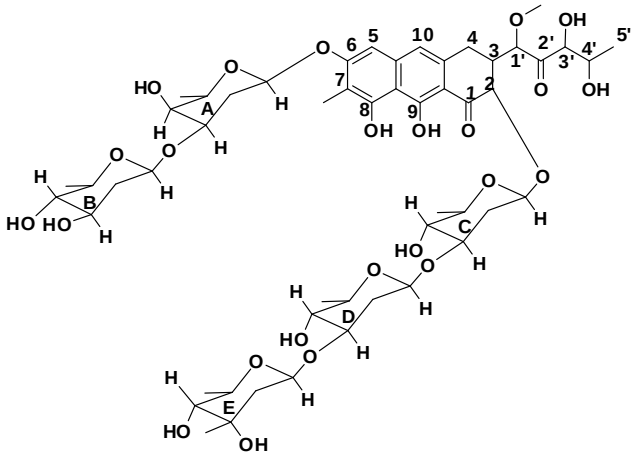

Figure 4: Chemical structure of Mithramycin.

has been renewed interest in aureolic acid class of compounds especially mithramycin, pertaining to new uses and activities such as inhibition of apoptosis and antiangiogenic activity [36]. Moreover clinical trials for mithramycin especially in cancer have also been started recently in USA with sponsorship of NCI [37,38] . This indicates that pharmaceutical researchers still have not lost hope for mithramycin, to be used as a drug candidate in future.

Although from the beginning mithramycin had always been presented as an antitumor drug candidate, as described in Introduction section, recently it has also been proposed as a potential candidate for developing new therapeutic drugs for various other disorders. Similarly, we endeavored to exploit mithramycin for anti-MRSA and anti-VRE activity. Extensive STN (Scientific and Technical Information Network) and other

Table 3: ^{1}H NMR Data of Mithramycin.

^{1}H Data of mithramycin (DMSO - d_6, 500 MHz)			^{1}H Data of mithramycin (DMSO - d_6, 500 MHz)		
Position	**δH**	**Multiplicity**	**Position**	**δH**	**Multiplicity**
1	-	-	1B	4.63	dd
2	4.64	d	2Ba	1.65	ddd
3	2.75	dddd	2Be	2.46	ddd
4	3.1	dddd	3B	4.9	ddd
4'	2.97	dddd	4B	4.71	dd
4a	-	-	5B	3.46	dq
5	6.87	s	6B	1.21	d
6	-	-	1C	4.99	dd
7	-	-	2Ca	1.73	ddd
CH_3-7	2.06	s	2Ce	2.63	ddd
8	-	-	3C	4.09	ddd
8-OH	9.81	s	4C	4.64	dd
9	-	-	5C	3.46	dq
9-OH	15.57	s	6C	1.21	d
10	6.82	s	1D	4.57	dd
10a	-	-	2Da	1.73	ddd
1'	4.59	d	2De	1.97	ddd
OCH_3-1'	Overlapped with solvent		3D	4	dt
2'	-	-	4D	4.99	dd
3'	5.17	d	5D	3.56	dq
4'	5.3	d	6D	1.16	d
5'	1.36	d	1E	4.89	dd
1A	4.96	dd	2Ea	1.5	dd
2Aa	1.97	ddd	2Ee	1.99	dd
2Ae	2.46	ddd	3E	-	-
3A	4	ddd	3E-CH_3	1.12	s
4A	4.71	dd	4E	4.57	d
5A	3.63	dq	5E	3.78	dq
6A	1.18	d	6E	1	D

Table 4: *In vitro* Antibacterial Activity of Mithramycin (MIC Expressed in µg/ml).

Test culture (n)	**Mithramycin**			**Linezolid**		
	MIC range	**MIC_{50}**	**MIC_{90}**	**MIC range**	**MIC_{50}**	**MIC_{90}**
S. aureus MRSA* **(15)**	0.125-0.250	0.125	0.25	2.00-8.00	4.00	4.00
S. aureus 209P MSSA **(1)**	0.125	-	-	4.00	-	-
E.faecalis VRE **(3)**	2.00	-	-	4.00	-	-
E.faecalis VSE **(1)**	2.00	-	-	4.00	-	-
E.faecium VRE **(2)**	1.00-16	-	-	2.00-4.00	-	-
E.faecium VSE **(1)**	4.00	-	-	2.00	-	-
Clinical *Enterococci* spp.VRE **(10)**	1.00-2.00	1.00	2.00	2.00-4.00	2.00	4.00
Bacillus spp**. (4)**	0.031-1.00	-	-	1.00-2.00	-	-
Gram Negatives [*E.coli* strains **(3)**, *Acinetobacter baumannii* strains **(2)**, *Pseudomonas* spp.**(3)**] **(8)**	>16	-	-	NT	NT	NT

MRSA: methicillin-resistant *Staphylococcus aureus*
MSSA: methicillin-sensitive *Staphylococcus aureus*
VRE: vancomycin-resistant enterococci
VSE: vancomycin susceptible enterococcus
n: number of test strains
*: including 13 clinical strains and two standard ATCC strains
NT: Not tested

literature searches carried out by us, showed that mithramycin has not been reported for activity against MRSA and VRE strains, making the present report as the first established report of mithramycin, exhibiting potent antibacterial activity against MRSA and VRE strains.

Development of a drug as an antibiotic needs mode of action studies along with *in vivo* susceptibility data. At present outcome of such studies for mithramycin cannot be predicted. However, considering the ever increasing cost of new drug discovery program all over the world, reports of challenges faced by Linezolid [20] and Daptomycin [21] and over all the grave problem of resistance of staphylococci and enterococci to these established drugs [16], this study can make scientists revisit the use of mithramycin scaffold, despite its toxicity and side effects.

As confirmed by *In vitro* studies, mithramycin has potent antibacterial activity. This may be one of the important multiple factors, facilitating dose reduction, required for treating infected animals as compared to high dose regimen of the compound required for the treatment of cancer. Moreover, cancer treatment being a long term process, frequency of occurrence of unpleasant

side effects of mithramycin is much more. In contrast, treatment for infectious diseases can be completed in limited period of time and toxic side effects of mithramycin can be minimized considerably.

Recently Nunez et al. [39] reported novel mithramycin analogue (demycarosyl-3D-β-d-digitoxosylmithramycin SK) with high antitumor activity and less toxicity. So alternatively, mithramycin can be used as a 'much needed potential scaffold' for synthesis of less toxic analogs which can also act as anti-MRSA drugs. As mithramycin is a known compound, the time and money required for new drug development in infectious disease area can be reduced substantially and in future mithramycin and its analogs would be new leads for the treatment of variety of Gram positive infectious diseases, associated with resistant strains of MRSA and VRE.

In conclusion, mithramycin obtained from *Streptomyces* sp. PM1129877, isolated from playa region of Thar Desert in Rajasthan, India, was purified and identified using spectroscopic methods. MIC estimations revealed that mithramycin exhibited anti-MRSA and anti-VRE activity, which was very potent compared to Linezolid. As regimen for infectious disease can be of limited period as compared to lengthy cancer treatment, authors propose and hope that in future mithramycin and its analogs could be potential candidates for developing a suitable lead for treatment of such diseases.

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