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Original Article

Microbiology Section

In-vitro Evaluation of Antimicrobial Activity of Nargenicin-A1 against Gram Positive Clinical Isolates and its Comparison with Various Antibiotics

SHEETAL GOUDA[1], BASAVARAJ V PEERAPUR[2], ABDUL K BAHADUR[3], VENKATESH R NAIK[4]

ABSTRACT

Introduction: The development of Multidrug Resistance (MDR) is a serious health problem, which demands the quest and development of many antibacterial agents. The class Actinomycetes represents best source for many antimicrobial agents. The present focus on Actinomycetes has yielded many antimicrobial agents including Nargenicin-A1. The Nargenicin-A1 belonging to class macrolides was found to have strong antibacterial activity against *Staphylococcus* and *Streptococcus*.

Aim: To determine the Minimum Inhibitory Concentration (MIC) of Nargenicin-A1 against clinically isolated aerobic gram positive bacteria and comparing its antimicrobial activities with various antibiotics.

Materials and Methods: A prospective, hospital-based, observational study was conducted at the Department of Microbiology, Raichur Institute of Medical Sciences, Raichur, Karnataka, India, from August 2015 to July 2016. All the clinical samples like pus, sputum, urine, ear swabs, wound swabs and body fluids received for culture and sensitivity testing at the Department of Microbiology during the study period was included. The antimicrobial activity was determined by measuring the MIC of Nargenicin-A1 by broth dilution method following standard procedure and the mean MIC was calculated. Pearson's coefficient of correlation was calculated to find out correlation between MIC of Nargenicin-A1 and MIC of various antibiotics effective against gram positive bacteria.

Results: The most common isolate was *Staphylococcus* followed by *Enterococcus* and *Streptococcus*. The least mean MIC of Nargenicin-A1 was observed for *Streptococcus* (0.017 µg/mL) followed by *S. aureus* (3.97 µg/mL), and the highest mean MIC value was recorded for *Enterococcus* (27.34 µg/mL). Among *Staphylococcus aureus*, the mean MIC value of Nargenicin-A1 for Methicillin Sensitive *Staphylococcus aureus* (MSSA), Methicillin Resistant *Staphylococcus aureus* (MRSA) and Vancomycin Resistant *Staphylococcus aureus* (VRSA) was 0.06 µg/mL, 0.12 µg/mL and 25 µg/mL, respectively. When compared Nargenicin-A1 with various antibiotics in terms of their MICs, the activity of Nargenicin-A1 was in close proximity to that of vancomycin and linezolid against MSSA, MRSA, and Enterococci and marginally with linezolid against VRSA.

Conclusion: Nargenicin-A1 exhibits strong antibacterial property against a broad spectrum of aerobic gram positive bacteria, including VRSA. The study revealed that Nargenicin-A1 can be considered as a potential alternative against MDR gram positive bacteria.

Keywords: Antimicrobial resistance, Minimum inhibitory concentration, *Staphylococcus*

INTRODUCTION

Medical intervention in infection mainly attempts to eradicate the pathogens by using substances obtained from microbes or chemically synthesised. These substances are collectively referred to as antimicrobial agents. Many years of use and misuse of these compounds have resulted in the resistance and appears to be an inevitable consequence [1]. Antimicrobial resistance has become a major global health problem. It has been the main subject of discussions in many scientific sessions during the past decade, but still, there are no indications that it is abating [2]. Resistance to antimicrobial agents can represent an enormous cost to the patient and the entire healthcare system. Further, the development of MDR microbes adds to the misery. The estimated economic cost due to antibiotic resistance in India (at INR 32 per standard unit of antibiotics) could be around INR 64,000-70,400 crores [3]. The antimicrobial resistance raises an alarm on the judicious use of antibiotics and stresses on the need for the quest of novel antimicrobials to combat the condition.

Development in technology has aided the discovery of many antibiotic producing microbes by genome mining, representing a shift from traditional antibiotics target [4]. Among the antibiotic producing microbes, the class Actinomycetes represents the best source for novel antibiotics [5,6]. Recent studies have focused on isolating new strains of Actinomycetes, which are known to synthesise many bioactive compounds [7-9]. Among the Actinomycetes, *Nocardia* species represent a new microbial source for novel antibiotics and many bioactive substances [10,11]. Many antimicrobial agents were isolated from nocardiae, including Nocardicin from *Nocardia uniformis*, Nargenicin-A1 from *Nocardia argentinensis*, Neocitreamycins from *Nocardia* strain (GO655), Nodusmycin from *Nocardia brasiliensis*, and Tubelactomycin from *Nocardia* sp.IMK703-102F1 [12-16]. However, the present study focuses on Nargenicin-A1 since comparing activities among Nocardicin, Nargenicin-A1, Tubelactomycin, and Nodusmycin indicate that Nargenicin-A1 is considerably more potent and active in-vitro [11,17,18].

Nargenicin-A1 is a 28-carbon macrolide with a tricyclic lactone containing an ether bridge with the chemical formula $C_{28}H_{37}NO_8$ and a molecular mass of 515.5953 g/moL and was discovered in the 1980s [13,19,20]. Nargenicin-A1 has been isolated from soil-dwelling microorganism *Nocardia argentinensis*, *Nocardia arthritidis*, and *Nocardia* CS682 strain [11,21,22]. The production of this macrolide can be enhanced by using synthetic biological

platform [23]. Nargenicin-A1 is found to be a potent anticancer agent and has shown inhibition properties against angiogenesis [24,25]. Nargenicin-A1 also has demonstrated strong antibacterial activity against *Staphylococcus, Streptococcus, Enterococcus* and *Clostridium* [22,26]. It was found to be more active in-vitro against *Staphyloccocus* strains but shows pronounced activity against *Streptococcus* [27]. Nargenicin-A1 exhibits stronger anti-MRSA activity than oxacillin, monensin, erythromycin, spiramycin, and vancomycin [22]. Although, the activities of Nargenicin-A1 against *Staphylococcus aureus (S. aureus)* strains are comparable to that of erythromycin, its cytotoxicity is remarkably lower than those of erythromycin and spiramycin against *S. aureus* [20,22]. Researchers have evaluated and compared Nargenicin-A1 with other drugs against *S. aureus,* however, the data on various isolates were missing. Moreover, the data supporting the biological activity of Nargenicin-A1 are scanty, and its comparison with various drugs against various isolates is still lacking. Hence, the present study aimed at determining the MIC of Nargenicin-A1 against clinically isolated aerobic gram positive bacteria and compared its activity with various antibiotics.

MATERIALS AND METHODS

A prospective, hospital-based, observational study was conducted from August 2015 to July 2016 at Bacteriology section in the Department of Microbiology, Raichur Institute of Medical Sciences, Raichur, Karnataka, India, after obtaining clearance from the Institutional Ethical Committee (IEC) (RIMS/IEC-33/2015 vide letter dated 13-07-2015). A non probability sampling method was applied and a total of 97 samples fulfilling the inclusion criteria were included in the study.

Inclusion criteria: All the clinical samples like pus, sputum, urine, ear swabs, wound swabs and body fluids received for culture and sensitivity testing at the Department of Microbiology during the study period was included.

Exclusion criteria: The samples which revealed no growth, or which revealed the growth of gram negative bacteria were excluded.

Out of 483 samples received during the study period, 197 samples revealed growth on blood agar and MacConkey agar. Gram stain was performed on these colonies. If the gram stain, confirmed the presence of gram positive bacteria, then these colonies were further subcultured on nutrient agar or blood agar to obtain pure growth. A total of 97 samples revealed the growth of 105 aerobic gram positive bacteria, and these 105 isolates were utilised for the study.

Study Procedure

Nargenicin-A1 was procured commercially from Allied Scientific Products and was tested on clinically isolated aerobic gram positive bacteria. The antimicrobial activity of Nargenicin-A1 was determined by measuring the MIC by broth dilution method.

Determination of MIC of Nargenicin-A1 by broth dilution method [28,29]

The broth dilution method is a quantitative technique for determining the MIC of antimicrobial agents. The highest dilution of the antimicrobial agent, which shows clear fluid with no developments of turbidity, was recorded as the MIC. The inoculum was prepared from a broth culture incubated for four hours. The density of the suspension is adjusted to approximately 10^8 colony forming units per milliliter (cfu/mL) by comparing its turbidity to McFarland 0.5 standard, which was prepared by adding 1% of 0.05 mL anhydrous barium chloride and a cold 1% of 9.95 mL solution of pure sulphuric acid.

Dilution of Nargenicin-A1 for *Staphylococcus* and *Streptococcus*

The dilution was started from 1 µg as the MIC was in the range of 0.1-0.2 µg/mL [22,30,31].

1000 µg was dissolved in 500 mL of autoclaved distilled water.

500 mL=1000 µg

1 mL=2 µg

So, 1 mL of the solution contains 2 µg of the Nargenicin-A1. The protocol for dilution for *Staphylococcus* and *Streptococcus* is shown in [Table/Fig-1] [32].

Tube no.	MH broth in mL	Nargenicin-A1 in serial dilution in mL	Discard in mL	Culture in mL	Final concentration of nargenicin-A1 (µg/mL)
1	1	1	-	0.1	1
2	1	1	-	0.1	0.5
3	1	1	-	0.1	0.25
4	1	1	-	0.1	0.125
5	1	1	-	0.1	0.063
6	1	1	-	0.1	0.031
7	1	1	-	0.1	0.016
8	1	1	-	0.1	0.008
9	1	1	-	0.1	0.004
10	1	1	1	0.1	0.002
11	1	-	-	0.1	-

[Table/Fig-1]: Protocol for dilution of Nargenicin-A1 for *Staphylococcus* and *Streptococcus*.
MH: Mueller hinton

Dilution of Nargenicin-A1 for *Enterococcus*

The dilution was started from 200 µg/mL as the MIC for *Enterococcus* was in the range of 75-50 µg/mL [26,31]. The protocol for dilution is shown in [Table/Fig-2].

Tube no.	MH broth in mL	Nargenicin-A1 in serial dilution in mL	Discard in mL	Culture in mL	Final concentration of vargenicin-A1 (µg/mL)
1	1	1	-	0.1	200
2	1	1	-	0.1	100
3	1	1	-	0.1	50
4	1	1	-	0.1	25
5	1	1	-	0.1	12.5
6	1	1	-	0.1	6.25
7	1	1	-	0.1	3.125
8	1	1	-	0.1	1.563
9	1	1	-	0.1	0.781
10	1	1	1	0.1	0.391
11	1	-	-	0.1	-

[Table/Fig-2]: Protocol for dilution of Nargenicin-A1 for *Enterococcus*.

1000 µg of Nargenicin-A1 was dissolved in 5 mL of distilled water

10 mL=4000 µg

1 mL=400 µg

The MIC was noted by visualising the tube with no visible turbidity. The MICs of various antibiotics were tested using the Vitek-2 system. Many studies compared Vitek-2 with Clinical and Laboratory Standards Institute (CLSI) guided broth dilution method for clinically significant aerobic bacteria, including Staphylococci, Streptococci, and Enterococci and showed categorical agreement that ranged from 94-100%, 95-98%, and 92-97%, respectively [33-35]. This indicates that Vitek-2 can be compared to the broth dilution method for determining antibiotic susceptibility patterns. In

the present study, the MIC of Nargenicin-A1 thus obtained by broth dilution method was compared with the MIC of various antibiotics obtained by the Vitek-2 system.

STATISTICAL ANALYSIS

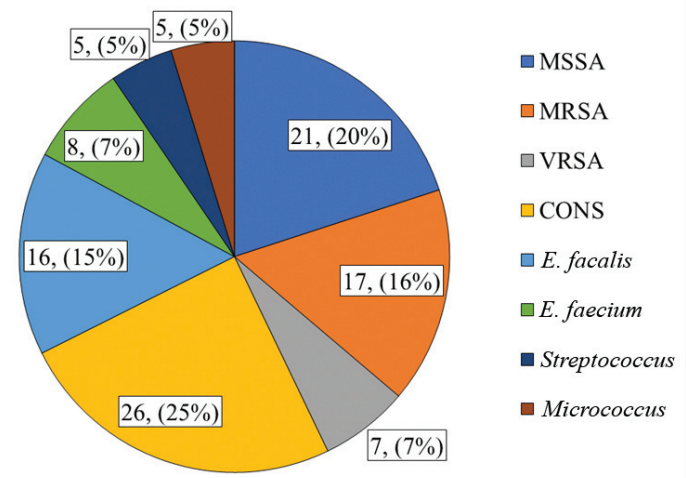
The MIC of Nargenicin-A1 and various antibiotics were determined. Pearson's coefficient of correlation (r) was calculated to know the correlation between MICs of Nargenicin-A1 and MICs of various antibiotics. The coefficient was determined separately for *Staphylococcus, Enterococcus* and *Streptococcus*. A positive correlation suggests that as the MIC of the test drug increased, the MIC of Nargenicin-A1 also increased. A negative correlation indicates that as the MIC of the test drug increased, the MIC of Nargenicin-A1 decreased. No correlation suggests that there is no variation in the MIC of Nargenicin-A1 with the increase or decrease in the MIC of the test drug.

RESULTS

A total of 97 samples showed growth of aerobic gram positive bacteria, which yielded 105 isolates for the study. The distribution of samples and the number of isolates obtained from various samples are shown in [Table/Fig-3]. The different gram positive isolates obtained from the study includes MRSA, Methicillin Sensitive *S. aureus* (MSSA), Coagulase Negative Staphylococci (CONS), *Enterococcus faecalis* (*E. faecalis*), *Enterococcus faecium* (*E. faecium*), *Streptococcus* and *Micrococcus* as shown in [Table/Fig-4]. The present study showed *S. aureus* (45) as the most common isolate.

Type of sample	No. of samples	No. of isolates
Pus	48	53
Urine	19	21
Sputum	9	10
Blood	11	11
Body fluids	3	3
Stool	7	7
Total	97	105

[Table/Fig-3]: Distribution of samples and the number of isolates obtained.



[Table/Fig-4]: Distribution of different gram positive bacteria obtained in this study.

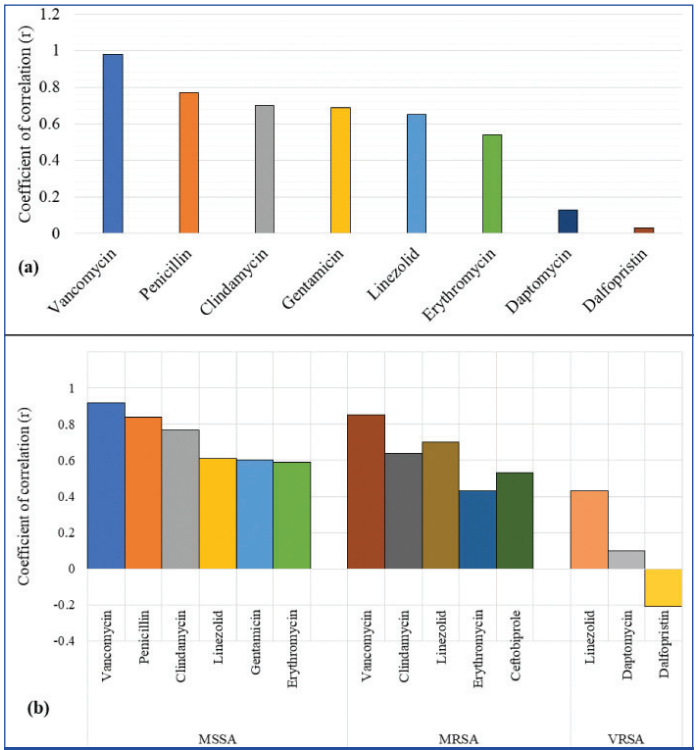
Mean MIC for different isolates: The MIC of Nargenicin-A1 was recorded for various isolates. The highest MIC was observed for *Enterococcus*, and the least was recorded for *Streptococcus*. The range of MIC and the mean MIC obtained for Nargenicin-A1 against different gram positive bacteria is shown in [Table/Fig-5].

Correlation of Nargenicin-A1 with various antibiotics against *S. aureus*: The MIC of Nargenicin-A1 was compared with MICs of various drugs active against *S. aureus.* The antibiotics tested include penicillin, oxacillin, ceftriaxone, clindamycin, erythromycin, gentamycin, vancomycin, ceftobiprole, daptomycin, linezolid, and dalfopristin. For *S.aureus*, the highest positive correlation was found with vancomycin (r=0.98); followed by penicillin (r=0.77), clindamycin (r=0.70), gentamycin (r=0.69) and linezolid (r=0.65). A weak positive correlation was noted with erythromycin (r=0.54), oxacillin (r=0.36), ceftriaxone (r=0.21), and ceftobiprole (r=0.17). However, daptomycin (r=0.13) and dalfopristin (r=0.03) showed no correlation with Nargenicin-A1 as shown in [Table/Fig-6a].

Bacteria	Range of MIC (µg/mL)	Mean MIC (µg/mL)
S. aureus	0.03-50	3.97
MRSA	0.06-0.2	0.12
MSSA	0.03-0.12	0.06
VRSA	12.5-50	25
CONS	0.03-0.2	0.06
Enterococcus	6.25-100	27.34
E. faecalis	6.25-25	14.45
E. faecium	12.5-100	53.13
Streptococcus	0.008-0.03	0.017

[Table/Fig-5]: Range of MIC obtained for Nargenicin-A1 for different gram positive bacteria.

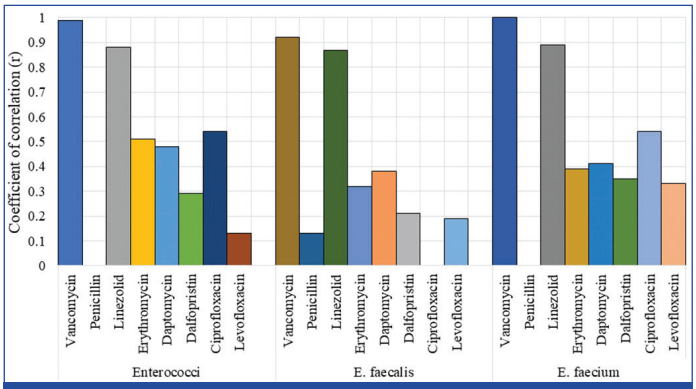
The correlation coefficient was further determined against MSSA, MRSA, and VRSA. For MSSA, the highest positive correlation was noted with vancomycin (r=0.92), followed by penicillin (r=0.84) and clindamycin (r=0.77). For MRSA, the highest positive correlation was noted for vancomycin (r=0.85) followed by linezolid (r=0.7). For VRSA, the highest positive correlation was noted for linezolid (r=0.43) the details of which are shown in [Table/Fig-6b].



[Table/Fig-6]: Coefficient of correlation of Nargenicin-A1 with various antibiotics against: (a) *S. aureus*; and (b) MSSA, MRSA, and VRSA.

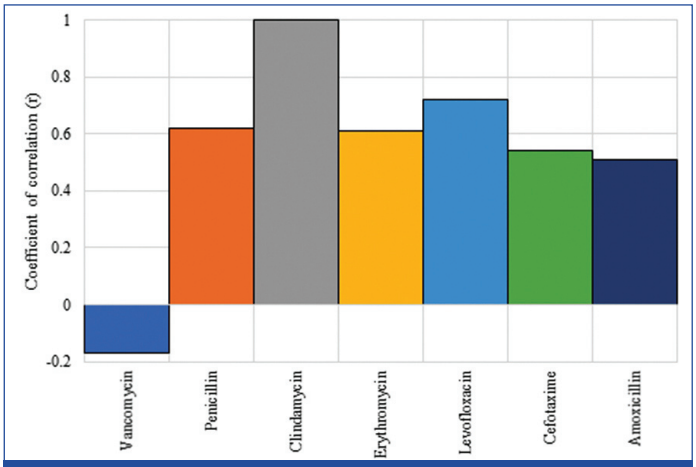
Correlation of Nargenicin-A1 with various antibiotics against *Enterococcus* species: The correlation coefficient was determined between the MICs of Nargenicin-A1 and MICs of various antibiotics active against Enterococci. The antibiotics tested include penicillin, erythromycin, ciprofloxacin, vancomycin, linezolid, daptomycin, levofloxacin and dalfopristin. As shown in [Table/Fig-7], the highest positive correlation was found with vancomycin (r=0.99) followed

by linezolid (r= 0.88). Weak positive correlation was found with ciprofloxacin (r=0.54), erythromycin (r=0.51) and daptomycin (r=0.48). No correlation was noted with dalfopristin (r=0.29), levofloxacin (r=0.13) and penicillin (r=0). For *E. faecalis* and *E. faecium*, the highest positive correlation was found with vancomycin followed by linezolid, the details of which is shown in [Table/Fig-7].



[Table/Fig-7]: Coefficient of correlation of Nargenicin-A1 with other antibiotics against Enterococci species.

Correlation of Nargenicin-A1 with various antibiotics against Streptococci: The coefficient of correlation was determined for MIC of Nargenicin-A1 with MIC of various antibiotics active against Streptococci. The drugs tested include penicillin, amoxicillin, clindamycin, cefotaxime, erythromycin, levofloxacin, and vancomycin. The highest positive correlation was found with clindamycin (r=1) followed by levofloxacin (r=0.72). Weak positive correlation was found with penicillin (r=0.62), erythromycin (r=0.61), cefotaxime (r=0.54), and amoxicillin (r=0.51). However, a negative correlation was noted with vancomycin (r=-0.17). The [Table/Fig-8] shows the details of the MIC of different antibiotics against Streptococci species.



[Table/Fig-8]: Coefficient of correlation of Nargenicin-A1 with other antibiotics against Streptococci species.

DISCUSSION

The present study showed *S. aureus* as the most common isolates 45 (42.9%). This may be because pus was the most common sample obtained 48 (49.5%) samples, and *S. aureus* is the major cause of pus-forming lesions [36-38].

The mean MIC obtained for *S. aureus*, MSSA, MRSA, and VRSA is 3.97, 0.06, 0.12, and 25 µg/mL respectively. The findings are similar to various studies conducted by Celmer WD et al., Magerlein BJ, Cho Ss et al., Li G et al., and Hong CY et al., further stating that Nargenicin-A1 has antibacterial activity against *S. aureus* [13,20,26,30,31]. However, the values of MICs obtained in the present study are slightly lower compared to other studies as shown in [Table/Fig-9] [13,20-22,26,30,31,39].

For *E. faecalis* and *E. faecium*, the mean MIC was 14.45 and 53.13 µg/mL, respectively. This was similar to a study conducted by Painter RE et al., further proving that Nargenicin-A1 exhibits antibacterial property against *Enterococcus* [39]. However, the value of MIC obtained in the present study was slightly lower as shown in [Table/Fig-9]. The mean MIC obtained for Streptococci was 0.017 µg/mL. This was similar to the study carried out by Pidot SJ et al., and Cho Ss et al., suggesting that Nargenicin-A1 has strong antibacterial activity against Streptococci [21,26].

The mean MIC obtained for the present study against various gram positive isolates was lower than the literature. This may be due to difference between the strains (biological variation) which contributes to 48% of the total variation in the MIC [40]. Interlaboratory and unexplained assay variations also have a substantial contribution of 10% and 42%, respectively [40].

Correlation of Nargenicin-A1 with various antibiotics against *S.aureus*

Nargenicin-A1 was compared with various antibiotics active against *S. aureus* in terms of their MICs. For MSSA and MRSA, the highest positive correlation was noted with vancomycin and linezolid, suggesting that as the MIC of vancomycin or linezolid increased, the MIC of Nargenicin-A1 also increased. This correlation indicates that Nargenicin-A1 can be considered as good as vancomycin and linezolid against MSSA and MRSA. For VRSA, a positive correlation was noted with linezolid suggesting that as the MIC of Nargenicin-A1 increased, the MIC of linezolid marginally increased. This correlation indicates that Nargenicin-A1 can be considered as an alternative to linezolid against VRSA. Similar findings in the literature show that Nargenicin-A1 exhibited stronger anti-MRSA activity than erythromycin, spiramycin, and vancomycin and demonstrated lower cytotoxicity compared to erythromycin and spiramycin [22].

Correlation of Nargenicin-A1 with various antibiotics against Enterococci and Streptococci

The highest positive correlation in terms of MICs for Nargenicin-A1 against Enterococci was found with vancomycin followed by linezolid,

S. No.	Author	Publication year	Place	MIC (µg/mL)					
				MRSA	MSSA	VRSA	CONS	*E. faecalis*	*Streptococcus*
1	Celmer WD et al., [13]	1979	Argentina	0.2	0.1	-	0.2-0.8	-	-
2	Magerlein BJ [20]	1984	USA	0.2	-	-	-	-	-
3	Hong CY et al., [31]	1997	Korea	0.6	0.13	-	0.08-0.13	-	-
4	Li G et al., [30]	2014	Germany	0.3	-	-	-	-	-
5	Cho Ss et al., [26]	2014	Korea	0.3	-	>80	-	>80	>80
6	Painter RE et al., [39]	2015	USA	-	-	-	-	>32	-
7	Sohng JK et al., [22]	2008	Korea	0.3	-	>80	-	>80	-
8	Pidot SJ et al., [21]	2019	Australia	-	-	-	-	-	0.5
9	Present study	2020	Raichur, India	0.12	0.06	25	0.06	14.45	0.017

[Table/Fig-9]: Comparison of mean MIC of Nargenicin-A1 obtained in this study with the literature [13,20-22,26,30,31,39].

suggesting that as MIC of vancomycin and linezolid increased, the MIC of Nargenicin-A1 also increased. This correlation suggested that Nargenicin-A1 can be considered as good as vancomycin and linezolid against Enterococci. Similar studies were conducted by Sohng JK et al., and Cho Ss et al., where Nargenicin-A1 demonstrated antibacterial property against Enterococcis [22,26]. For Streptococci, the highest positive correlation was found with MIC of clindamycin and followed by levofloxacin suggesting that, Nargenicin-A1 can be considered as good as clindamycin or levofloxacin against Streptococci. Similar study was conducted by Pidot SJ et al., and Cho Ss et al., where antibacterial activity was demonstrated against Streptococci [21,26]. However, comparison of Nargenicin-A1 with various antibiotics active against Enterococci and Streptococci are lacking. This necessitates further comparative analysis on the same.

Limitation(s)

The present study aimed at evaluating the effect of Nargenicin-A1 against clinically isolated aerobic gram positive bacteria. However, the isolates obtained were limited to *S. aureus, E. faecalis, E. faecium*, CONS, Streptococci and Micrococci. Although, the MIC of Nargenicin-A1 was determined by broth dilution method, the MICs of various antibiotics were obtained from VITEK-2 system. The difference in the MICs noted by these two methods could have contributed to minor variation in the data.

CONCLUSION(S)

The present study suggested that Nargenicin-A1 exhibits strong antibacterial properties against a broad spectrum of aerobic gram positive bacteria. However, comparative analysis of Nargenicin-A1 with various antibiotics active against gram positive bacteria is lacking and needs additional studies. Further extensive research and clinical trials on Nargenicin-A1 are required to know pharmacokinetics or pharmacodynamics to optimise the dosage and monitor adverse drug reactions.

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PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Microbiology, Karwar Institute of Medical Sciences, Karwar, Karnataka, India.
2. Professor and Head, Department of Microbiology, Raichur Institute of Medical Sciences, Karnataka, India.
3. Assistant Professor, Department of Microbiology, Raichur Institute of Medical Sciences, Karnataka, India.
4. Associate Professor, Department of Microbiology, Raichur Institute of Medical Sciences, Karnataka, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Sheetal Gouda,
Assistant Professor, Department of Microbiology, Karwar Institute of Medical Sciences, M.G. Road, Karwar, Karnataka, India.
E-mail: sheetalkgouda@gmail.com

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