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# ASSESSMENT OF ANTIBIOTIC PRODUCTION POTENTIAL IN TWO ACTINOMYCETES AGAINST VANCOMYCIN RESISTANT ENTEROCOCCI

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## ABSTRACT

Crude extracts of two actinomycetes ACT24 and ACT25 were analysed for antibacterial activities against wide range of bacterial strains. The extracts showed antibacterial activities against both Gram-negative and Gram-positive test bacteria with zone of inhibition ranging between 2 and 15mm. The minimum inhibitory concentration of the extracts against the test bacteria ranged from 1.2mg/ml – 4.2 mg/ml. The extracts of ACT24 and ACT25 exhibited concentration time dependent killing of Vancomycin resistant enterococcus with a 4.62Log10 and 3.46Log10 reduction in viable counts respectively. ACT24 ethylacetate extract exhibited prolonged post antibiotic effect against vancomycin enterococci giving a significantly more favorable effect than reference compound which is most commonly used systemic antibiotic chloramphenicol. This assessment reveals that antibiotic from ACT24 has potential to inhibit the vancomycin resistant enterococci and this can be exploited for further treatment in future by analysing the structure of the compound.

**KEYWORDS: Actinomycetes, antibiotic production, time kill assay and antibacterial activity, VRE**

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# INTRODUCTION

Natural products are novel potential chemical structures possessing antimicrobial activity. At least three of every four current antibacterial agents are related in some way to natural products. The filamentous bacteria of the order *Actinomycetales* (actinomycetes) produced more than 9000 biologically active molecules out of which more than 60 pharmaceutical agents have widely used in the field of medicine. *Streptomyces* are a prolific source of secondary metabolites yielded many antibiotics, more than 80% antibiotics available in the market are from *Streptomyces*, including streptomycin, neomycin, tetracycline andchloramphenicol [1]. Actinomycetes are originally considered to be an intermediate group between bacteria and fungi but now are recognized as prokaryotic organisms which are widely distributed in soil, water and found colonizing plants[2,3]. As the search for producers of novel compounds continues, it becomes apparent that many Streptomycetes from different environments produce same compound. Marine compounds have been attracting the attention of scientists for more than 50 years. Recently new species and new genera of marine actinomycetes have been described[4]. Antimicrobial resistance is resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it. Resistant microorganisms (including bacteria, fungi, viruses and parasites) are able to withstand attack by antimicrobial drugs, such as antibacterial drugs (e.g. antibiotics), antifungals, antivirals, and antimalarials, so that standard treatments become ineffective and infections persist, increasing the risk of spread to others. The use and misuse of antimicrobial drugs accelerates the emergence of drug-resistant strains. New resistance mechanisms emerge and spread globally threatening our ability to treat common infectious diseases, resulting in death and disability of individuals who until recently could continue a normal course of life. Without effective anti-infective treatment, many standard medical treatments will fail or turn into very high risk procedures[5]. Actinomycetes are important sources of new bioactive compounds which have diverse clinical effects and are active against many pathogenic organisms. Actinomycetes and their bioactive compound show antibacterial and antimicrobial against various pathogens and multi drug resistant pathogens e.g. Vancomycin-Resistant *Enterococci* (VRE), Methicillin-Resistant *Staphylococcus aureus*, *Shigella dysenteriae*, *Klebsiella* sp. and *Pseudomonas aeruginosa* etc[6]. The problem with VRE is that it may be resistant to many antibiotics limiting the choice of antibiotics available to treat the infection. It may make some VRE infection more difficult to treat. The need for new, safe and effective antimicrobial agent is the major challenge to the pharmaceutical industry and the need for increased exploration of previously unexplored habitats for new actinomycete taxa has become a major focus in the search for the next generation of pharmaceutical agents especially with the increasing trend in development of antibiotic resistant in microbial pathogen [7,8]. In this paper, we reported on the antibacterial potentials of actinomycete extract produced by two actinomycete isolates belonging to the genera *Streptomyces* and isolated from the aquatic environment of south India as part of our ongoing research of new antimicrobial compounds.

# MATERIALS AND METHODS

### *(i) Test actinomycetes*

Two actinomycetes strains were isolated from Pichavaram mangrove, Cuddalore, Tamil Nadu and the isolates were tentatively identified as genera Streptomyces. The organisms were maintained on agar slants and in 20% glycerol at -80°C. The test actinomycetes suspensions were prepared by suspending a loopful of pure actinomycetes colony in 10 ml sterile physiological saline, vortexed to homogenize and stored at 4°C until ready for use[3]. This suspension was used as actinomycetes inoculants in all cultivations.

### *(ii) Clinical isolate*

*Klebsiella pneumoniae*, *Bacillus subtilis*, *Proteus vulgaris*, *Enterococcus faecalis* and *Staphylococcus aureus* Vancomycin resistant *Enterococcus faecalis*

***(iii) Preparation of test bacteria***

The test bacteria were grown in nutrient broth and incubated at 37°C for 24 h. The cells were centrifuged at 7000 rpm, washed with sterile physiological saline and standardized to $OD_{600nm}$ 0.1 and stored at 4°C until ready for use [8].

***(iv)Preparation and inoculation of fermentation medium***

The fermentation broth was prepared as follows per litre: 10 g starch, 4 g yeast extract, 2 g peptone g/l .The medium was aliquoted in 500 ml Erlenmeyer flasks and sterilized by autoclaving at 121°C and 15 lb for 15 min and allowed to cool after which 100 µl of the standardized actinomycetes suspension were used to inoculate the flasks and incubated at 27°C on a shaker at 300 rpm for 10 days [1].

***(v)Extraction of antibiotic metabolites from fermentation cultures***

The extracts of the fermentation products were recovered from the culture filtrate by solvent extraction using ethyl acetate. Ethyl acetate was added to the filtrate in the ratio 1:1 (v/v) and shaken vigorously for complete extraction. The ethyl acetate phase that contains the crude antibiotic metabolite was separated from the aqueous phase and concentrated in vacuum at 60°C using a rotary evaporator. The residue obtained was weighed and reconstituted in 50% ethyl acetate[9].

***(vi) Antibacterial susceptibility test***

The antibacterial activities of the crude ethyl acetate extracts were determined using agar well diffusion method. Twenty millilitres of sterilized molten Mueller Hinton agar was seeded with 50 µl of standardized test bacteria, swirled gently and aseptically poured into Petri dishes and allowed to solidify. Sterile cork borer was used to bore wells in the plate, and 30 µl of the crude ethyl acetate extract at a concentration of 10 mg/ml was then carefully dispensed into the bored holes. The extract was allowed to diffuse for about 1 h before incubating aerobically at 37°C for 24 h. The presence of a zone of inhibition around each well was indicative of antibacterial activity. Control experiments were carried out by loading 30 µl of 50% ethyl acetate in place of the extracts [10].

***(vii)Determination of minimum inhibitory concentration (MIC)***

The sterile plastic, disposable microtitre plates with 96 flat-bottom wells were used. The medium used in the plates were prepared at double the final strength to allow for a 50% dilution once the inoculum and solvents/ or antimicrobial were added. A 100 µl volume of double strength Muller Hinton broth was introduced into all the 96 wells and varying concentrations of the antibiotic were added in decreasing order along the wells after which wells were loaded with 50 µl of the test organism suspension. The plates were then incubated at 37°C for 18-24 h. Results were read using a micro titre plate reader (BIO-TEK model 680) at 490 nm.

***(viii) Determination of the time kill assay***

The time-kill assay of the crude extracts were determined by checking the rate of bacterial cell death with respect to time using test bacteria. The resultant cell suspension was diluted 1:100 with fresh sterile nutrient broth and used to inoculate 50 ml volume of Mueller Hinton broth incorporated with extract at multiples of the MIC to a final cell density of $5 \times 10^5$ cfu/ml. Controls consisted of extract free Mueller Hinton broth inoculated with test bacteria[11]. Colony counts were immediately performed before incubation. Time–kill tubes were then placed in a 37°C incubator (room air, humidified) on a shaking platform at 150 rpm, and colony counts were performed on each tube at 3, 6, 9, 12, 15, 21 and 24 h. All tests were performed in duplicate [12].

***(ix) Determination of post antibiotic effect (PAE)***

The PAE was induced by exposure to multiples of the MIC of culture extract for 1 h. For PAE testing, a mid-logarithmic-phase culture of the vancomycin enterococci was prepared. Tubes containing 5 ml of nutrient broth with extract were inoculated with approximately $5 \times 10^6$ CFU/ml. Growth control with inoculum but no actinomycete extract was included in each experiment. Inoculated test tubes were placed in a shaking water bath at 35°C for an exposure period of 1 h. At the end of the exposure period, cultures were diluted 1:1000 in prewarmed broth to remove the extract by dilution. Extract removal was confirmed by comparing growth curves of a

control culture containing no antibiotic to another containing extract 0.01 times the exposure concentration. Viability counts were determined before exposure and immediately after dilution (0 h) and then every 2 h for up to 10 h or until turbidity of the tube reached 1 McFarland standard. The PAE was calculated according to the Craig and Gudmundsson formula

PAE = *T* _ *C*,

where *T* refers to the time required for the treated culture to recover by 1 log10 CFU greater than that observed immediately after drug removal (time zero) and *C* refers to the corresponding recovery time observed for the untreated control[13].

# RESULTS

### 1.*Antibacterial activities of the crude extracts*

The results of the antibacterial activities of the crude extracts of the fermentation are shown in Table 1. Total of 2 actinomycete extracts were screened against 6 test bacteria. ACT24 extract showed antibacterial activities against five of the test bacteria with zone of inhibition ranging between 9-15 mm, while ACT 25 extract was active against all the six test bacteria with zone of inhibition varying between 2 and 12 mm. It revealed that ACT 24 produced more potent antibacterial compound as it exhibited larger inhibition zone than ACT 25 extract, although it showed activity against all clinical isolates. The decreasing rate of discovery of novel drugs from established terrestrial sources has motivated the evaluation of new sources of chemically diverse bioactive compounds. Natural products are a boundless source for important novel compounds having antagonistic activity against pathogenic organisms. Marine environment covers almost 70% of the earth surface. The filamentous actinomycetales species produces over 10000 bioactive compounds, 7600 derived from Streptomyces and 2500 from the so called rare actinomycetes (rare actino) species, represent the largest group (45%) of bioactive microbial metabolites[14]. In 2013 Gebreselema *et al*., isolated actinomycetes from water and sediments. He observed a wide range of zone of inhibition in the primary screening[15].

**Table 1**
***Antibacterial activities of crude extracts of the two test actinomycetes***

| Test organisms | Zone of inhibition (mm) | |
|---|---|---|
| | ACT 24 | ACT 25 |
| ***Staphylococcus aureus*** | 15 | 12 |
| ***Bacillus subtilis*** | 12 | 8 |
| ***Eschericia coli*** | 12 | 8 |
| ***Enterococcus faecalis*** | 14 | 10 |
| ***Klebsiella pneumonia*** | - | 2 |
| ***Serratia marcescens*** | 9 | 5 |
| ***VRE*** | 10 | 6 |

***+activity, - no activity, VRE Vancomycin resistant enterococi clinical isolate.***

### *2. Minimal inhibitory concentration*

The minimum inhibitory concentration (MIC) of the two extracts are presented in Table 2. The MIC of the extract ACT24 ranged from 1.2 to 3.1 mg/ml. For extract of ACT25 MIC varied between 2.2 and 4.2 mg/ml. More yield of crude extract was produced through fermentation. During secondary screening, 2 crude extracts namely ACT24 and Act25 showed wide range of inhibition against VRE strain. The bioactivity of the isolates was dissimilar between Gram positive and Gram negative bacterial strains. The results clearly demonstrated that crude extract of ACT24 highly active when compared to ACT25. The MIC varied among the tested isolates against VRE. These results were similar with the report of Sibanda *et al* (2010) [12].

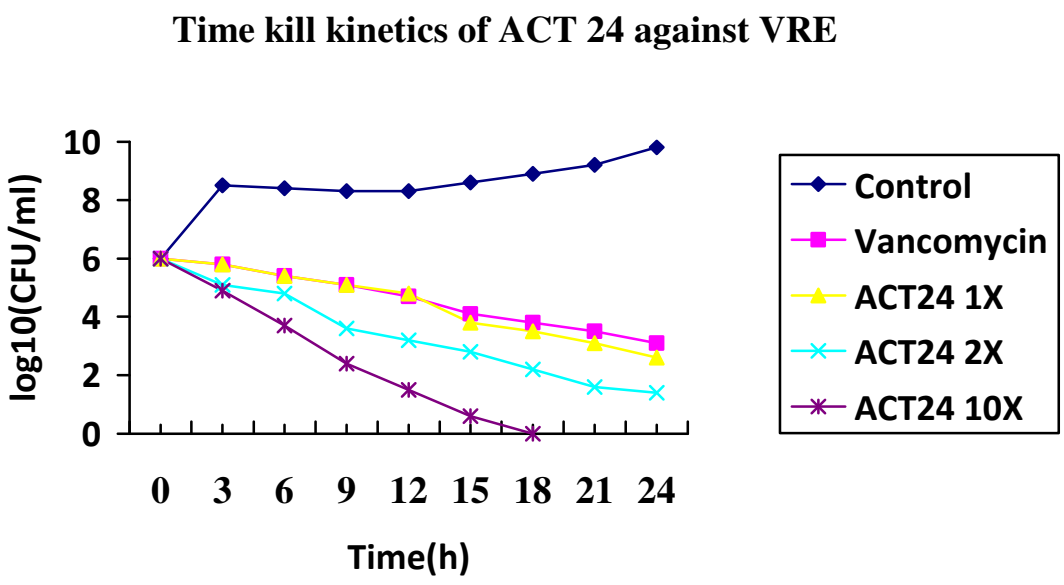
**Table 2**
***Minimal inhibitory concentration of crude extract of two actinomycetes***

| Test organisms | Minimal inhibitory concentration (mg/ml) | |
|---|---|---|
| | ACT24 | ACT25 |
| *Staphylococcus aureus* | 1.2 | 2.2 |
| *Bacillus subtilis* | 2.5 | 2.8 |
| *Escherichia coli* | 2.4 | 2.6 |
| *Enterococcus faecalis* | 2.3 | 2.7 |
| *Serratia marcescens* | 2.7 | 4.2 |
| *VRE* | 3.1 | 4.0 |

### *3. Time kill assay*

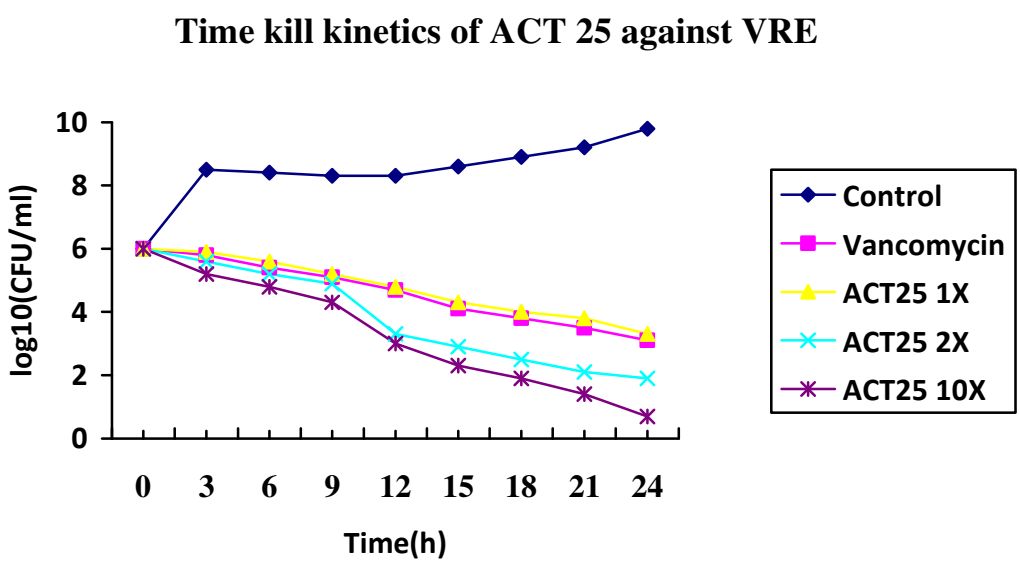
The time kill studies were conducted for antimicrobial compounds at 1X, 2X, 10X concentration from ACT24 and ACT25 against VRE. Graph 1 and Graph 2 shows the log10 change in viable count of VRE at different concentration of extract and comparison against vancomycin. The rate kill of bacteria is varied in concentration of extract and different time exposure. The viable cell count of ACT24 and ACT25 extract against VRE was observed from 5.8 - 2.6 and 5.9- 3.3 respectively. Aqueous extract of ACT24, the log reduction ranged between 5.1 Log10 and 1.4 Log10 cfu/ml in three hours time interval upto 24 h with 2X MIC. VRE was showed completely reduced growth at 18h exposure against ACT24 whereas in ACT 25 at 24h with the 10X MIC values.

**Graph 1**
***Time kill kinetics of ACT24 and vancomycin against VRE at multiples of MICs***



**Graph 2**

***Time kill kinetics of ACT25 and vancomycin against VRE at multiples of MICs***

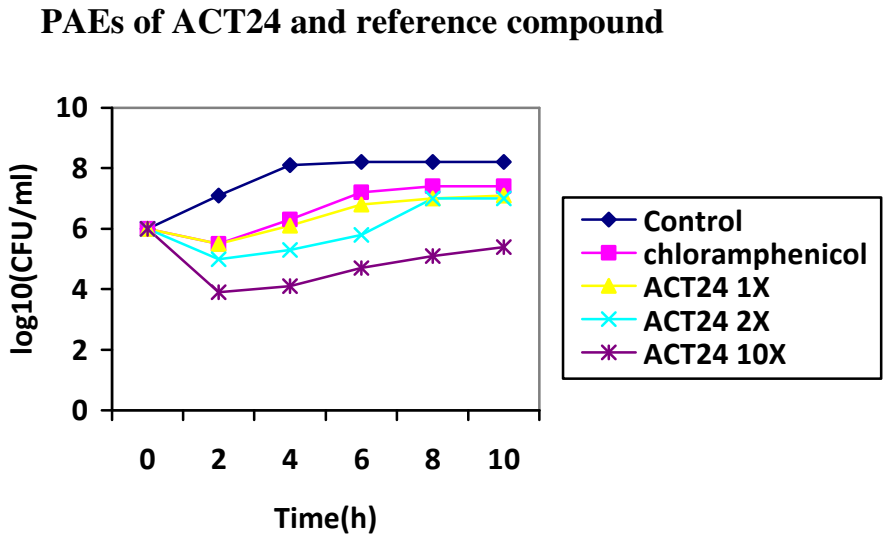



***4. Post antibiotic effect***

We investigated the PAE of ACT24 extract against VRE. As shown in Graph. 3, actinomycete antibiotic at 1X, 2X and 10X MIC exhibited a concentration dependent PAE against VRE strain. ACT24 ethylacetate extract exhibited prolonged PAEs against VRE giving a significantly more favorable PAE (4 - 6 h) with 10X MIC than reference compound the most commonly used systemic antibiotic chloramphenicol.

**Graph 3**

***Post antibiotic effect of ACT24 at their different MICs concentration***




The PAE of antimicrobial agents against *Legionella* spp. has studied by Martin and Pendland[16]. In 1998 they found that both clarithromcyin and its metabolite have a considerable PAE against pathogens. This PAE appears to be strain-specific, in that two of the isolates had a PAE roughly double that of the others. Again, the combination of both antibiotics had shorter PAEs than either drug alone for three of the four organisms [16].

## CONCLUSION

Aquatic natural actinomycete antibiotic from ACT24 possesses potent *in vitro* antibacterial activities against VRE when compared to other reference compound and ACT25. Actinomycete antibiotic demonstrated potent concentration-dependent bactericidal kinetics against VRE. The speed killing kinetics of ACT24 against VRE shows that ACT24 has potentials as sources of lead compounds in new drug discovery. This natural antibiotic even at low concentration effective against drug resistant strains. This potential of the compound is opens up for further characterization and identification of the compound.

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