African Journal of Pharmacy and Pharmacology Vol. 4(12), pp. 908-916, December 2010
Available online http://www.academicjournals.org/ajpp
ISSN 1996-0816 ©2010 Academic Journals

Full Length Research Paper

In vitro time-kill studies of antibacterial agents from putative marine *Streptomyces* species isolated from the Nahoon beach, South Africa

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Accepted 13 December, 2010

We assessed the antibiotic production potentials of ten putative *Streptomyces* strains isolated from the Nahoon beach and their antibacterial activities against a wide range of bacteria including reference strains, environmental strains and clinical isolates. The minimum inhibitory concentrations (MICs) of the crude ethyl acetate extracts ranged from 0.039 to 10 mg/mL and the least minimum bactericidal concentration (MBC) demonstrated was 0.625 mg/mL against a reference strain *Staphylococcus aureus* ATCC 6538. Time kill kinetics of all extracts revealed bacteriostatic and bactericidal activities. Average log reductions in viable cell counts for all the extracts ranged from 0.86 log_{10} and 3.99 log_{10} cfu/mL after 3 h interaction and 0.01 log_{10} and 4.86 log_{10} after 6 h interaction at MIC, 2 × MIC, 3 × MIC and 4 × MIC concentrations. Most of the extracts were speedily bactericidal at 3 × MIC and 4 × MIC resulting in over 50% elimination of most of the test bacteria within 3 and 6 h interaction. Our findings suggest that the marine *Streptomyces* isolated from the Nahoon beach have tremendous potential as sources of new antibacterial compounds.

Key words: Time-kill, antibacterial compounds, Marine streptomyces, Nahoon beach.

INTRODUCTION

Naturally occurring substances of microbial origin have provided a continuing source of antibiotics and medicines since the origin of man. The side effects experienced with available drugs, the misuse and over use of antibiotics have led to a wide spread resistance of antibiotics among human, animal and plant pathogens (Mahyudin, 2008). This has `prompted researchers to explore for novel drugs with little or no side effects that could be used to treat infections caused by resistant bacteria pathogens.

Marine *Streptomyces* are potential candidates for novel natural products and they exhibit a unique metabolic diversity and great potential in producing novel compounds. They produce approximately two-thirds of all known antibiotics of microbial origin (Hou et al., 2006). They are well known as a rich source of antibiotics and bioactive molecules and are thus considered to be a rich biotechnological resource and continue to be an excellent source of novel compounds (Hou et al., 2006).

Marine ecosystems contain several unique features that set them apart from other aquatic ecosystems, the main factor being the presence of dissolved compounds in seawater, particularly sodium chloride. For marine microorganisms, their cellular adaptation to moderate and high salt content is a fundamental biological process needed for survival and growth. It is postulated that marine *Streptomyces* have different characteristics from those of their terrestrial counterparts and, therefore, might produce different types of bioactive compounds (Mahyudin, 2008). The immense diversity of this habitat

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along with it's under exploitation is the fundamental reason for attracting researchers towards it for discovering novel metabolite producers.

Interest in the importance of marine organisms as a source of new substances is growing. With marine species comprising approximately half of the total global biodiversity, the sea offers an enormous resource for novel compounds (de Vries and Beart, 1995), and it has been classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity (Gerwick and Bernart, 1993). As part of our ongoing programme to exploit marine environment natural products for drug discovery and development; marine *Streptomyces* was isolated from the sea waters and rock scrapings of the Nahoon beach and screened for antibacterial activity. In this paper, we report on the in vitro time kill characteristics of antibacterial agents produced by some marine Streptomyces isolated from the Nahoon beach as part of our exploration for new bioactive compounds from marine organisms in the South Africa marine environments.

MATERIAL AND METHODS

Sample collection

Samples were collected from the Nahoon beach, which is a coastal shore of the Indian Ocean located in East London in the Eastern Cape Province of South Africa at the geographical co-ordinates 32.99°S and 27.95°E. Water samples were collected from knee level depths as the water washes in from the ocean, while sediment samples were collected from dug-outs of about 2 ft deep and no more than 20 m near the edge of the ocean using a spade. The rock scrapings were collected from rocks not more than 50 m to the edge of the water. Sediment samples were collected in sterile polythene bags, and water sample and rock scrapings were collected in sterilized 1 liter bottles. All samples were transported in cooler boxes to the laboratory for analyses as described elsewhere (Ogunmwonyi, 2008).

Test bacteria

The test bacteria that were used in this study include reference, environmental as well as clinical isolates. The typical reference strains were as follows:

i. Gram-positive: *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis ATCC 29212*, *Streptococcus pyogens* ATCC 10389, *Bacillus cereus* ATCC 10702, *Bacillus pumilus* ATCC 14884, *Acinetobacter calcaoceuticus* UP and *Acinetobacter calcaoceuticus* subsp *anitratus* CSIR.
ii. Gram-negative: *Escherichia coli* ATCC 8739, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 7700, *P. aeruginosa* ATCC 19582, *Proteus vulgaris* ATCC 6830, *Proteus vulgaris* CSIR 0030, *Enterobacter cloacae* ATCC 13047, *Serratia marcescens* ATCC 9986, *Klebsiella pneumoniae* ATCC 10131 and *K. pneumoniae* ATCC 4352.
iii. Environmental isolates: *K. pneumoniae*, *Bacillus subtilis*, *Shigella flexineri*, *Salmonella* sp, *Staphylococcus epidermidis*, *P. aeruginosa*, *P. vulgaris*, *Enterococcus faecalis*, *Escherichia coli*, *S. aureus* and *Bacillus stearothermophillus* were kindly provided by Dr. AO Olaniran of the University of KwaZulu-Natal, Durban, South Africa.

Clinical isolates: Staphylococcal strains isolated from septic wound abrasion: *S. aureus* OKOH1, *S. aureus* OKOH2A, *S. aureus* OKOH3, and *Staphylococcus sciuri* OKOH2B with Gene Bank accession numbers Eu244633, Eu244634, Eu244635 and Eu244636 respectively.

Preparation of test bacteria inocula

All test bacteria were grown in Nutrient broth (Biolab, Merck) and incubated at 37°C for 24 h and a total of 10 mL of the pure culture was centrifuged to pellet out the cells, washed twice with sterile physiological saline and the suspension adjusted to optical density 0.1 at 600 nm which is equivalent to a cell population of about 10^6 cells/mL on the McFarland standard (Donay et al., 2007). Bacteria suspension was stored in test tubes and refrigerated at 4°C. These test bacteria were checked for viability a day before use and where cell densities are reduced below the 10^6 cells/mL standard fresh suspension are re-prepared.

Source of *Streptomyces*

The *Streptomyces* used in this study were isolated from the Nahoon beach (Ogunmwonyi, 2008), amongst many other actinomycetes species, and stored in the culture collection of the applied and environmental microbiology research group (AEMREG), University of Fort Hare. The actinomycetes isolates were culturally characterized based on colour, dryness, rough, with irregular or regular margin, and generally convex colony morphology, tough leathery colonies, branched vegetative mycelia, and when present, aerial mycelia and spore formation as described by Ghanem et al. (2000) and grouped into generic morphotypes including the *Streptomyces*. Ten *Streptomyces* isolates that showed most potent activity based on zones of inhibition were selected for this study. The isolates were coded as follows; NB003, NB008, NB009, NB012, NB017, NB022, NB046, NB063, NB078 and NB084.

Preparation of *Streptomyces* suspension

The test *Streptomyces* suspensions were prepared by suspending a loopful of pure *Streptomyces* colony in 2 mL sterile normal saline, vortexed to homogenize and stored at 4°C until ready for use. This suspension was used as *Streptomyces* inoculants in all cultivations.

Fermentation and preparation of crude ethyl acetate extracts

Fermentation for production of antibiotic and subsequent extraction of the antibiotics was done as described by Ilic et al. (2007) with modification. Yeast malt extract broth (YMB) was prepared and 20 mL dispensed into 100 mL Erlenmeyer flask capacity, sterilized, allowed to cool and inoculated with 0.5 mL *Streptomyces* isolate suspension and incubated at 28°C for 48 h at 230 rpm. About 500 mL of YMB was prepared in 1L Erlenmeyer flask and inoculated with the 48 h old pre-culture of *Streptomyces* isolate and incubated for 10 days at 28°C at 230 rpm. At the end of the incubation period, the culture was harvested by centrifugation at maximum speed for 15 min. The culture supernatant was extracted twice with equal volumes of ethyl acetate (1:1 v/v) and vaporized to dryness in a rotary evaporator at 50°C. The extract was re-constituted in 50% filter sterilized methanol to obtain the desired concentration at every stage of screening.

Cultivation media composition

Yeast malt broth (YMB) was used for the cultivation of the test

Streptomyces and had the following composition: [Yeast extract 4 g/l (biolab, Merck), Malt extract broth10 g/l (Conda, Pronadisa), Glucose 4 g/l (Saarchem, Merck)]. Nutrient agar (NA) [(meat extract 1 g/l, yeast extract 2 g/l, peptone 5 g/l, sodium chloride 8 g/l, agar 15 g/l (biolab, Merck)]. Nutrient broth (NB) [(Meat extract 1 g/l, yeast extract 2 g/l, peptone 5 g/l, sodium chloride 8 g/l (biolab, Merck)]. These media were prepared according to the manufacturer's instructions.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts

The MIC and MBC was determined using the broth micro dilution method as described by Eloff (1998), using test bacteria that have been standardized to optical density of 0.1 at 600 nm. Extracts were re-suspended in 10% filter sterilized methanol to obtain a concentration of 40 mg/mL. Using a 96-well microtitre plate, 100 µl double strength nutrient broth was dispensed into every well and labeled appropriately. Fifty microliter of extract was added into well 3, mixed carefully using pipette and diluted serially along the rows to row 11. Fifty microliter (50 µl) of the standardized test bacteria was incorporated into the 96-well microtitre plate. Plates were covered and incubated at 37°C for 18 to 24 h. Control wells were loaded with nutrient broth and extracted with no bacteria added (wells 1 A-H), broth and bacteria with no extract (wells 12 A-H), 10% MeOH, broth and bacteria (wells 2 A-H) to ensure that 10% MeOH constitution solvent had no inhibitory effect on the test bacteria. Each extract was assayed in triplicate against test bacteria. Biolab micro plate reader (Model 680 S/N 19138) was used to quantify the optical density of the reactants in each well.

A graph of optical densities of wells contents was plotted against extracts concentrations. From the graph the MICs of extracts against test bacteria were estimated. Also, to complement this assay, bacterial growth was confirmed by adding 50 µl of 0.2 mg/mL solution of 2[4-iodophenyl]3[4-nitrophenyl]-5-phenyl-2H-tetrazolium chloride (INT) in each test well, and the plate was incubated further for at least 1 h at 37°C, to ensure adequate colour development. The lowest concentration in which there was a definite decrease in colour was taken as the MIC of that extract, for that particular organism. The MBC of active extracts was determined by streaking on Nutrient Agar plates from wells that showed growth inhibition. Streaking was done in triplicates per well that showed growth inhibition to enhance sensitivity and accuracy. The concentration of extract in the wells where there was no growth on plate was considered as the MBC.

Determination of rate of kill

The method of Spangler et al. (1997) was adopted for the time-kill assay against some selected susceptible bacteria. Viable counts of the test bacteria were first determined. Initial inocula of 10^5 to 10^6 cfu/mL were prepared from 100 µl aliquots of test bacteria in normal saline and this was verified by performing colony counts. Eighty microliter (80 µl) volume of suspension of known cell density of selected test bacteria was added to 10 mL of Nutrient broth in McCartney bottles of known concentration (relative to MIC) of each of the extracts. Time kill assay of each extract against the selected test bacteria were determined using the following extract concentrations: MIC, 2 × MIC, 3 × MIC and 4 × MIC and the reactants bottles were incubated in a rotary incubator at 37°C. The time kill kinetics were determined at 0, 3 and 6 h. Exactly 0.5 mL volume of the reaction mixture was withdrawn at the appropriate time and transferred to 4.5 mL of nutrient broth recovery medium containing 3% sodiumthioglycolate or 3% "Tween-80" to neutralize the effects of the extracts carry-overs from the test suspensions and diluted serially in sterile nutrient broth. Approximately 100 µl aliquots of each dilution were plated out for viable counts by pour plate technique. Each experiment was done in duplicate, and the mean of two almost identical results was calculated. Only plates yielding 30 to 300 colonies were selected for counting. Data were analyzed by expressing growth as the $\log_{10}$ colony forming unit per milliliter (cfu/mL). For the Time-kill end point determination; bacteriostatic activity was defined as a reduction of 0 to 3 $\log_{10}$ cfu/mL, and bactericidal activity was defined as a reduction of ≥ 3 $\log_{10}$ cfu/mL at 3 and 6 h compared to that at 0 h (Ellof, 1998). McCartney bottles containing broth and test organism without extract was used as growth control in each experiment.

RESULTS

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The results of minimum inhibitory concentration (MIC) of the crude extracts against the susceptible bacteria are presented in Table 1. Extract of NB003 exhibited the MIC in the range of 0.1562 to 5 mg/mL, while NB008 had MIC varying between 0.039 and 2.5 mg/mL. The MIC of NB009 extract was revealed to range between 0.039 mg/mL and 1.25 mg/mL and that of NB012 ranged from 0.1562 to 2.5 mg/mL. For NB017, MIC ranged from 1.25 to 2.5 mg/mL, while for NB022 it varied between 0.3125 mg/mL and 5 mg/mL For extracts of NB046, NB063, NB078 and NB084, MICs ranged between 0.039 and 5 mg/mL; 0.078 and 0.625 mg/mL; 0.078 and 10 mg/mL; and 0.3125 and 2.5 mg/mL, respectively. The MBCs of the extracts ranged between 0.625 and >10 mg/mL, with most of the extracts having MBCs of >10 mg/mL (Table 2).

Time kill assay

The time-kill profile of the crude extracts of the test *Streptomyces* are as shown in Table 3. All extracts exhibited varying degrees of bactericidal and bacteriostatic activities depending on the test bacteria. A significant decrease in mean viable count of isolates was observed at each time interval. Results are presented as $\log_{10}$ cfu/mL change in the viable colony number. Mean log reduction in viable cell count for NB003 range between 2.18 $\log_{10}$, 2.48 $\log_{10}$, 2.61 $\log_{10}$ and 3.09 $\log_{10}$ cfu/mL after 3 h interaction in MIC, 2 × MIC, 3 × MIC and 4 × MIC, respectively and between 2.36 $\log_{10}$, 2.56 $\log_{10}$, 2.80 $\log_{10}$ and 3.15 $\log_{10}$ cfu/mL after 6 h interaction in MIC, 2 × MIC, 3 × MIC and 4 × MIC, respectively. For NB008 Log reduction in viable cell count varied from 1.7 $\log_{10}$, 2.48 $\log_{10}$, 2.67 $\log_{10}$ and 3.05 $\log_{10}$ cfu/mL after 3 h of interaction at MIC, 2 × MIC, 3 × MIC and 4 × MIC, respectively while at 6 hr interaction it was revealed as 0.62 $\log_{10}$, 2.53 $\log_{10}$, 2.69 $\log_{10}$ and 3.15 $\log_{10}$ at MIC, 2 × MIC, 3 × MIC and 4 × MIC respectively. For NB009, NB012, NB022, NB046, NB063, NB078 and NB084 extracts, the log reduction in viable cell densities ranged between -0.51 $\log_{10}$ and 4.86 $\log_{10}$. The utmost

Table 1. Minimum inhibitory concentrations (MICs) of the crude extracts.

Test bacteria	Gram reaction	MIC (mg/mL)									
		NB003	NB008	NB009	NB012	NB017	NB022	NB046	NB063	NB078	NB084
E. coli ATCC 8739	-	0.625	N/A	0.1562	N/A	N/A	N/A	N/A	N/A	N/A	N/A
E. coli ATCC 25922	-	2.5	0.156	N/A	0.625	1.25	0.625	N/A	0.625	N/A	N/A
P. aeruginosa ATCC 19582	-	2.5	1.25	N/A	N/A	N/A	N/A	0.625	0.1562	N/A	N/A
S. aureus ATCC 6538	+	2.5	N/A	N/A	0.3125	N/A	0.625	1.25	0.1562	N/A	1.25
E. faecalis ATCC 29212	+	N/A	0.625	N/A	1.25	N/A	N/A	0.625	ND	N/A	0.3125
B. cereus ATCC 10702	+	2.5	0.625	0.078	0.3125	1.25	0.625	0.078	N/A	0.078	0.3125
B. pumilus ATCC 14884	+	1.25	0.625	0.625	0.625	N/A	0.3125	1.25	0.3125	0.625	1.25
P. aeruginosa ATCC 7700	-	2.5	2.5	N/A	N/A	N/A	N/A	0.625	0.3125	N/A	N/A
E. cloacae ATCC 13047	-	5	2.5	N/A	1.25	N/A	0.3125	N/A	0.625	1.25	N/A
K. pneumoniae ATCC 10031	-	0.3125	2.5	0.3125	0.3125	2.5	0.625	N/A	0.1562	0.625	0.3125
K. pneumoniae ATCC 4352	-	0.625	2.5	0.1562	0.3125	2.5	0.625	0.3125	N/A	0.3125	0.3125
P. vulgaris ATCC 6830	-	2.5	1.25	0.625	0.625	N/A	N/A	1.25	N/A	0.078	N/A
P. vulgaris CSIR 0030	-	N/A	2.5	N/A	N/A	N/A	N/A	0.625	0.078	0.3125	N/A
S. marcescens ATCC 9986	-	0.625	1.25	N/A	N/A	N/A	N/A	0.625	0.078	0.625	N/A
A. calcaoceuticus UP	+	0.625	1.25	0.625	0.1562	N/A	2.5	0.625	N/A	0.078	0.625
A. calcaoceuticus subsp *anitratus* CSIR	+	0.625	0.3125	N/A	0.3125	N/A	N/A	0.039	0.3125	0.3125	0.1562
K. pneumoniae KZN	-	5	N/A	N/A	N/A	N/A	N/A	0.625	0.3125	0.625	N/A
B. subtilis KZN	+	1.25	0.625	1.25	0.625	N/A	1.25	N/A	0.1562	0.1562	N/A
S. flexineri KZN	-	N/A	N/A	0.625	1.25	N/A	N/A	N/A	N/A	N/A	N/A
Salmonella sp KZN	-	0.1562	0.078	N/A	N/A	N/A	N/A	1.25	N/A	10	N/A
S. epidermides KZN	-	1.25	N/A	N/A	0.625	N/A	5	N/A	ND	N/A	N/A
P. aeruginosa KZN	-	N/A	1.25	0.3125	2.5	N/A	N/A	N/A	0.3125	5	N/A
P. vulgaris KZN	-	0.3125	1.25	0.625	N/A	N/A	N/A	N/A	N/A	0.3125	N/A
E. faecalis KZN	-	N/A	N/A	0.078	N/A	N/A	N/A	N/A	N/A	N/A	2.5
Escherichia coli KZN	-	2.5	1.25	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S. aureus KZN	+	0.3125	2.5	N/A	0.3125	N/A	1.25	0.625	N/A	1.25	N/A
S. aureus OKOH1	+	5	1.25	N/A	N/A	ND	2.5	N/A	N/A	ND	0.625
S. aureus OKOH 2A	+	N/A	N/A	N/A	0.3125	N/A	5	1.25	0.625	N/A	N/A
S. sciuri OKOH 2B	+	1.25	0.039	1.25	N/A	N/A	0.625	0.3125	N/A	N/A	N/A
S. aureus OKOH 3	+	2.5	0.3125	1.25	0.3125	1.25	0.3125	0.039	N/A	0.625	0.625
M. kristinae	+	0.3125	0.625	0.039	0.625	N/A	1.25	0.3125	ND	0.3125	0.625
M. luteus	+	0.1562	0.625	N/A	N/A	N/A	0.3125	N/A	N/A	0.625	N/A

ND- not determined; N/A – Not applicable.

Table 2. Minimum bactericidal concentrations (MBCs) of the crude extracts.

Test bacteria	Gram reaction	MBC (mg/mL)									
		NB003	NB008	NB009	NB012	NB017	NB022	NB046	NB063	NB078	NB084
E. coli ATCC 8739	-	>10	N/A	>10	N/A	N/A	N/A	N/A	N/A	N/A	N/A
E. coli ATCC 25922	-	>10	>10	N/A	>10	>10	>10	N/A	>10	N/A	N/A
P. aeruginosa ATCC 19582	-	10	10	N/A	N/A	N/A	N/A	5	2.5	N/A	N/A
S. aureus ATCC 6538	+	10	N/A	N/A	0.625	N/A	10	5	N/A	N/A	>10
E. faecalis ATCC 29212	+	N/A	10	N/A	>10	N/A	N/A	2.5	ND	N/A	>10
B. cereus ATCC 10702	+	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
B. pumilus ATCC 14884	+	>10	>10	>10	>10	N/A	>10	>10	>10	>10	>10
P. aeruginosa ATCC 7700	-	10	5	N/A	N/A	N/A	N/A	2.5	1.25	N/A	N/A
E. cloacae ATCC 13047	-	>10	>10	N/A	>10	N/A	>10	N/A	>10	>10	N/A
K. pneumoniae ATCC 10031	-	>10	>10	>10	>10	>10	>10	N/A	>10	>10	>10
K. pneumoniae ATCC 4352	-	>10	>10	>10	>10	>10	>10	>10	N/A	>10	>10
P. vulgaris ATCC 6830	-	>10	>10	>10	>10	N/A	N/A	>10	N/A	>10	N/A
P. vulgaris CSIR 0030	-	N/A	>10	N/A	N/A	N/A	N/A	>10	>10	2.5	N/A
S. marcescens ATCC 9986	-	>10	10	N/A	N/A	N/A	N/A	5	5	>10	>10
A. calcaoceuticus UP	+	5	10	5	>10	N/A	5	2.5	N/A	>10	2.5
A. calcaoceuticus subsp *anitratus* CSIR	+	5	2.5	N/A	10	N/A	N/A	2.5	>10	>10	1.25
K. pneumonia KZN	-	10	N/A	N/A	N/A	N/A	N/A	1.25	2.5	2.5	N/A
B. subtilis KZN	+	10	>10	2.5	>10	N/A	>10	N/A	>10	>10	N/A
S. flexineri KZN	-	N/A	N/A	2.5	2.5	N/A	N/A	N/A	N/A	N/A	N/A
Salmonella sp KZN	-	10	5	N/A	N/A	N/A	N/A	5	N/A	>10	N/A
S. epidermides KZN	-	10	N/A	N/A	>10	N/A	>10	N/A	ND	0	N/A
P. aeruginosa KZN	-	N/A	2.5	5	5	N/A	N/A	N/A	1.25	>10	N/A
P. vulgaris KZN	-	10	2.5	2.5	N/A	N/A	N/A	N/A	N/A	10	N/A
E. Faecalis KZN	-	N/A	N/A	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A
E. coli KZN	-	10	2.5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S. aureus KZN	+	10	5	N/A	5	N/A	5	2.5	N/A	>10	N/A
S. aureus OKOH1	+	10	2.5	N/A	N/A	ND	10	N/A	N/A	ND	>10
S. aureus OKOH 2A	+	N/A	N/A	N/A	5	N/A	>10	2.5	2.5	N/A	N/A
S. sciuri OKOH 2B	+	5	2.5	5	N/A	N/A	5	2.5	N/A	N/A	N/A
S. aureus OKOH 3	+	5	2.5	2.5	10	>10	>10	5	N/A	5	10
M. kristinae	+	1.25	2.5	2.5	2.5	N/A	10	1.25	ND	2.5	>10
M. luteus	+	2.5	2.5	N/A	N/A	N/A	5	N/A	N/A	10	N/A

ND – not determined; N/A – Not applicable.

Table 3. Antibacterial Time-kill profile of the crude extracts of the fermentation products.

Extracts	Test bacteria	MIC (Log_{10} kill)		2 × MIC (Log_{10} kill)		3 × MIC (Log_{10} kill)		4 × MIC (Log_{10} kill)	
		3 h	6 h	3 h	6 h	3 h	6 h	3 h	6 h
NB003	*P. vulgaris* KZN	2.18	2.36	2.48	2.56	2.61	2.80	3.09	3.15
	S. sciriu OKOH 2B	1.76	1.06	1.97	1.43	2.16	2.57	2.55	2.80
	K. pneumoniae ATCC 4352	1.10	-0.97	1.25	-0.12	1.38	1.92	1.41	2.24
NB008	*E. coli* ATCC 25922	1.38	-1.0	2.01	0.36	2.65	2.65	3.04	3.08
	S. sp. KZN	1.55	-0.89	1.16	1.14	1.65	1.97	1.78	2.63
	E. faecalis ATCC 29212	1.70	0.62	2.48	2.53	2.67	2.69	3.05	3.15
NB009	*S. sciriu* OKOH 2B	2.64	0.19	2.71	0.72	3.42	1.06	3.58	1.37
	B. cereus ATCC 10702	1.39	-2.81	1.47	-0.74	3.01	1.27	3.46	2.64
	P. aeruginosa KZN	1.73	1.20	2.26	2.04	2.38	2.11	2.58	2.58
	S. aureus ATCC6538	2.03	2.11	2.05	2.17	2.18	2.25	2.50	3.39
NB012	*K. pneumoniae* ATCC 10031	1.39	1.87	1.39	2.18	1.56	2.36	1.59	2.39
	B. pumilus ATCC 14884	1.52	2.10	1.97	2.39	2.40	3.04	3.69	3.79
NB022	*S. aureus* ATCC 6538	1.52	2.10	1.97	2.39	2.40	3.06	3.69	3.79
	M. luteus	1.5	1.49	1.72	1.72	3.44	2.55	3.99	4.38
NB046	*E. faecalis* ATCC 29212	1.53	-0.83	1.53	0	2.19	0.26	2.31	0.60
	Salmonella sp. KZNk.	1.14	-2.4	1.50	0.55	2.59	2.70	3.07	3.74
NB063	*S. aureus* OKOH 2A	1.44	1.26	1.65	2.14	1.81	2.47	3.92	4.86
	P. aeruginosa ATCC 19582	2.71	2.63	3.01	3.03	3.04	3.21	3.59	3.78
	S. marcescens ATCC 9986	1.32	-1.51	2.59	-0.51	2.63	-0.35	2.75	-0.22
NB078	*K. pneumoniae* ATCC 4352	1.39	-0.81	1.73	0.19	2.42	1.97	2.72	2.8
	P. vulgaris CSIR 0030	0.86	-2.16	1.10	-0.89	1.30	-0.79	1.30	0.01
	S. aureus OKOH 3	2.10	-2.10	2.67	-1.83	3.02	1.47	3.04	1.01
NB084	*E. faecalis* ATCC 29212	1.26	-1.32	2.30	0.183	2.34	2.61	3.41	3.45
	A.calcaoceuticus subsp *anitratus* CSIR	2.35	0.51	2.49	0.63	2.71	1.05	3.25	1.27

reductions in cell densities were achieved by NB063, NB022 and NB046 for *S. aureus* OKOH 2A (clinical isolate), *Micrococcus luteus* and *Salmonella* sp. KZN (environmental isolates) with the average reduction in viable cell of 4.86 $\log_{10}$, 4.38 $\log_{10}$ and 3.74 $\log_{10}$, respectively.

DISCUSSION

The biological activities (MIC and MBC) of the crude extracts emphasizes that the extracts are active against gram-positive and gram-negative bacteria. The MICs values exhibited by all extracts in this study ranged between 0.039 mg/mL and 10 mg/mL. These were relatively higher than the MIC values obtained from marine *Streptomyces* strain Merv 1996 (El-Gendy et al., 2008a) against *B. subtilis* ATCC 6051, *S. aureus* ATCC 6538 and *M. luteus* with MICs of 0.0036, 0.0008 and 0.002 mg/mL, respectively; *Streptomyces* sp. AZ-NIOFD1 isolated from River Nile water, Egypt (Atta et al., 2009) with MIC range of 0.0117 mg/ml–0.03125 mg/ml; and marine *Streptomyces* sp. Merv 8102 (El-Gendy et al., 2008b).

NB008 extract exhibited antagonistic activity against *Staphylococcus sciuri* OKOH 2B (a clinical isolate) with MIC of 39 µg/ml, which is slightly higher than the CLSI (2006) MIC breakpoint for clindamycin (≤ 0.5 µg/ml), tetracycline (≤ 4 µg/ml), ciprofloxacin (≤ 1 µg/ml) and chloramphenicol (≤ 8 µg/ml), but lower than the breakpoint for sulfonamides (≤ 256 µg/ml) against *Staphylococcus* spp. Also, NB063 extract had MIC of 78 µg/ml against *Proteus vulgaris* CSIR 0030 and *Serratia marscens* ATCC 9986, while extracts of NB063 had MIC of 312 µg/ml against *Pseudomonas aeruginosa* ATCC 7700 and *P. aeruginosa* KZN (environmental isolate), and 156 µg/ml against *P. aeruginosa* ATCC 19582. These values are generally higher than MIC interpretive standards of gentamicin (≤ 4 µg/ml), amikacin (≤ 16 µg/ml), ciprofloxacin (≤ 1 µg/ml), levofloxacin (≤ 2 µg/ml), ceftazine (≤ 8 µg/ml), cefepime (≤ 8 µg/ml), imipenem (≤ 4 µg/ml) and meropenem (≤ 4 µg/ml) against non-enterobacteriaceae. A similar trend was observed for NB009 extract, which had MIC of 78 µg/ml against *Bacillus cereus* ATCC 10702 and an environmental isolate (*Enterococcus faecalis* KZN). This MIC is higher than the MIC break point of ampicillin (≤ 8 µg/ml), tetracycline (≤ 4 µg/ml), erythromycin (≤ 0.5 µg/ml), ciprofloxacin (≤1 µg/ml) and chloramphenicol (≤8 µg/ml) against *Enterococcus* spp, however, this value is lower than the CLSI intermediate interpretive standard of fosfomycin (128 µg/ml) against *Enterococcus faecalis* (CLSI 2006). Hence, considering that the antibacterial compounds reported in this study are in the crude form, the relatively low MICs observed in the crude extracts in comparison with those of standard antibiotics suggest that these extracts could be very important sources of potent antibacterial compounds.

In general, some extracts of these putative *Streptomyces* had antibacterial activities against Gram positive bacteria, especially *staphylococci*. *Staphylococci* are among the most commonly encountered pathogens in clinical practice. *S. aureus* is a major cause of nosocomial infections, food poisoning, osteomyelitis, pyoarthritis, endocarditis, toxic shock syndrome, and a broad spectrum of other disorders (Sonavane et al., 2007; Schmidt et al., 2009). In recent years, there has been an alarming increase in nosocomial staphylococcal infections by strains with multiple drug resistance (Gouldet al., 2009; Elward et al., 2009). At present, this situation is leading to the evaluation of staphylococcal pathogens potentially resistant to any available antibiotic (Saha et al., 2008; Schmidt et al., 2009). Therefore, the result of this study may suggest that the extracts of the species possess compounds with antimicrobial properties which can be used as antimicrobial agents in new drugs for therapy of infectious diseases in human.

Time-kill studies have been used to investigate numerous antimicrobial agents. They are also often used as the basis for *in vitro* investigations into pharmacodynamic drug interactions. They provide descriptive (qualitative) information on the pharmacodynamics of antimicrobial agents. The time kill assay of the crude extracts of the test S*treptomyces* isolates gave variable kinetics among susceptible bacteria tested (Table 3). Both bactericidal and bacteristatic activities were demonstrated by the extracts. The extracts exhibited a partially concentration-dependent killing profile. Although, literature on time kill kinetics of marine *Streptomyces* is scarce, time kill kinetics of a marine bacterium against clinical methicillin resistant *S. aureus* (MRSA) isolate has been reported by Isnansetyo and Kamei (2003). In their study, bactericidal activity was demonstrated to be much higher than vancomycin therefore, time kill studies of these marine *Streptomyces* proposes their possible outcome in *in vivo* studies.

Extracts of NB008 was bactericidal with 4 × MIC against *Enterococcus faecalis* ATCC 29212 at 3 h and a slight increase in activity at 6 hr interaction. However, a bacteristatic activity was observed at lower concentrations. Watanakunakorn and Tisonel (1982), reported time kill kinetics of the combination of N-Formimidoyl' thienamycin (MK0787) a stable derivative of thienamycin, derived from *Streptomyces cattleya* with gentamicin or with tobramycin against *Enterococci*. In their study a combination of N-Formimidoyl' thienamycin with gentamicin or with tobramycin showed over 95% bactericidal activity against forty-seven strains of *Streptococcus faecalis* tested. Therefore extracts of NB008 compound or its derivative either single or in combinational therapy with other antibiotics suggest potential use against enterococcal infections. NB008 showed bacteristatic action against *Salmonella* sp at all concentrations tested. However, stronger bacteristatic

activity was observed with higher concentrations (3 × MIC and 4 × MIC) and longer time. It can be assumed that at higher concentration of this extract, aggressive bactericidal activity against *Salmonella* sp would be achieved.

NB063 exhibited bacteriostatic activity with MIC at 3 and 6 h against *P. aeruginosa* ATCC 19582. Furthermore, bactericidal activity was demonstrated with 2 × MIC, 3 × MIC and 4 × MIC at 3 and 6 h interactions. *P. aeruginosa* is an important pathogen associated with serious nosocomial infections such as pneumonia and sepsis (Tam, 2005). It is also associated with multiple mechanisms of resistance to various antimicrobial agents (Tam, 2005). Treatment of pseudomonal infections often presents a challenge to clinicians and combination therapy is commonly used to prevent the emergence of resistance (Tam, 2005). However, from the Time-kill profile among all extracts and susceptible bacteria tested in this study, NB063 showed the best time kill profile against *P. aeruginosa* ATCC 19582 in the sense that bactericidal activity was observed with 2 × MIC with a stepwise increase in bactericidal activity with concentration and time. Although, NB009 exhibited a strong bacteristatic activity against an environmental strain of *P. aeruginosa* KZN, NB063 is the only extract that demonstrated bactericidal activity with 2 × MIC concentrations. NB063 promises to be an important agent against pseudomonal infections.

Anti-pneumococcal activities were demonstrated by the extract from NB012 (against *K. pneumoniae* ATCC 10031), as well as extracts from NB003 and NB078 (against *K. pneumoniae* ATCC 4352). Time kill profile of anti-pneumococcal activities of some antibiotics has been reported (Pankuch et al., 1996) but the anti-pneumococcal time kill profile of marine *Streptomyces* is limited. These findings appear to be the first report of anti-pneumococcal time kill profile of extracts of marine *Streptomyces* (Table 3). This extracts (NB003, NB012 and NB078) promises to be important drug candidates for the development of anti-pneumococcal antibiotics. The re-growth phenomenon exhibited by some extracts against certain bacteria could be attributed to instability of the crude extracts in the growth medium.

Conclusions

This study suggests that the Nahoon beach promises to be a source of *Streptomyces* isolates producing antibacterial compounds that could be important in the search for leads for new antibiotics. The emergence and dissemination of antibacterial resistance is well documented as a serious problem worldwide. It is anticipated that due to the antibacterial profile and characterization of the crude extracts, putative marine *Streptomyces* isolated from Nahoon Beach promises to be useful in the discovery of new antibiotics. Although, time-kill kinetics was used to monitor the rate of kill of the extracted bioactive compounds against individual bacteria, there is limited information in the literature on imaging of time-kill kinetics at the cellular level. Thus future studies may focus on this area to broaden our knowledge and understanding of the mechanisms involved in this process.

ACKNOWLEDGEMENT

We are grateful to the National Research Foundation (NRF) of South Africa for funding this study.

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