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Antimethicilin resistance agents from marine actinomycetes from soil sediments of Lagos Lagoon

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated the antimicrobial efficacy of Actinomycetes isolated from lagos marine environment. GC-MS studies identified rifamycin as well as tubelactomicin as the inhibitory peptides.

Details on Page 198

ABSTRACT

Objective: To evaluate the isolation of actinomycetes strains with potential for producing antimicrobials with high methicilin resistance capability.

Methods: The soil samples were collected from four different locations of Lagos lagoon. The *Actinomycetes* were isolated from the samples by serial dilution using spread plate method. Isolates were selected based on their cultural characteristics as well as their Gram reaction and phenotypically and molecularly characterized *Streptomyces* sp. Isolates were inoculated in starch casein and Kuster's broth media and secondary metabolites were screened for antimicrobial activity against the following microorganisms: methicillin resistant *Staphylococcus aureus*, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 29522, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans*, *Enterococcus faecalis* ATCC 29212. Coagulase-negative staphylococci isolated from HIV patients were also used (*Staphylococcus warneri*, *Staphylococcus xylosus* and *Staphylococcus epidermidis*). The antimicrobial metabolites of the isolates were identified using gas chromatography-mass spectrometer.

Results: Extracts from isolates ULS12 and ULS13 showed antimicrobial activity against methicillin resistant *Staphylococcus aureus* while ULK3 inhibited *Candida albicans* only. The gas chromatography-mass spectrometer data analysis showed the antibiotic profile of these isolates.

Conclusions: The isolates ULS12 and ULS13 were found to display the highest antimicrobial activity against the test organisms and could be a potential source of new antibiotics.

KEYWORDS

Marine actinomycetes, *Streptomyces*, Antimethicillin resistance, Molecular identification, Morphological characteristics, GC-MS

1. Introduction

Many pathogenic bacteria are fast developing resistance to existing antibiotics. Hence, the urgency in the need for new drugs becomes apparent to control the incidence of infections caused by these antibiotic resistant pathogens as well as treat cancer related diseases which could be life threatening[1].

Methicillin resistance in *Staphylococcus aureus* (*S. aureus*) has been recognized globally since it was first reported in the United Kingdom by British scientists in 1961[2,3]. However, there has been increased difficulty combating methicillin resistant *S. aureus* because of emerging resistance to available antibiotics[4]. To solve the problem of antibiotic-resistant pathogens by periodic replacement of existing antibiotics with novel antibiotics, unique environments need to be constantly exploited for novel bioactive compounds. Bioactive compounds synthesized by microorganisms seem to be the most promising source of novel antibiotics[5].

Amongst the prokaryotes, members of the phylum Actinobacteria, particularly those which belong to the genus *Streptomyces* have been recognized as prolific sources of novel bioactive metabolite with a wide

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spectrum of activities[6,7].

The search for novel natural products have focused on the terrestial environment but in recent times, the marine environment, which remained unexplored with regard to isolation of antibiotic producing actinomycetes have now been considered as a new source of novel drugs. However, evidence in literature has shown that indigenous actinomycetes of the marine environment in West Africa have not been explored for their antibiotic production potentials. Therefore, the marine actinomycetes diversity of West Africa could be a potential source of novel antimicrobial compounds that can combat rapidly emerging drug-resistant pathogens that have became issues of important public health concern in Nigeria and world wide.

2. Materials and methods

2.1. Sample collection and isolation of Actinomycetes

Soil samples were collected from different locations of Lagos lagoon using pre-sterilized grab. The samples were kept in sterile polythene bags and transported immediately to the laboratory. They were air-dried for 2 weeks after which the *Actinomycetes* were isolated by serial dilution using spread plate method on Starch Casein and Kuster's Agar supplemented with 80 µg/mL of cycloheximide to prevent fungal growth[8]. The plates were incubated at 28 °C for 1-2 weeks. Isolates were selected based on their cultural characteristics as well as their Gram reaction and subcultured. Pure cultures were maintained on nutrient agar slants at 4 °C[9].

2.2. Biochemical characterization of isolates

Biochemical studies were carried out on the suspected actinomycetes isolates using API 20A kit (Biomerieux, France). The tests were carried out according to the manufacturer's instructions, incubated at 28 °C for 24-48 h and were later read. All the positive and negative tests were recorded on the result sheet. Other biochemical tests such as starch hydrolysis and casein hydrolysis were carried out using standard methods[10].

2.3. DNA extraction, amplification and sequencing

DNA was extracted from the isolates and stored at -20 °C. The primers S-C-Act-0235-a-S-20 5'CGC GGC CTA TCA GCT TGG TTG 3' and S-C-Act-0878-a-A-19 5'CCG TAC TCC CCA GGC GGG G 3', specific for Actinobacteria were used to amplify an 640-bp stretch of the 16S rRNA gene of all the strains using PCR method[10]. The PCR conditions were an initial denaturation stage at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60.5 °C for 45 seconds, extension at 72 °C for 50 seconds and a final extension at 72 °C for 5 min. Negative controls with no DNA template were included in all PCR experiments. Amplification was detected by agarose gel electrophoresis and UV fluorescence after ethidium bromide staining and prufied PCR products were sequenced (ABI 3730 DNA Analyzer) and sequences run on basic local alignment search tool for identification of the isolates[11].

2.4. Screening of secondary metabolites for antimicrobial activity

A loopful of each pure actinomycete culture was inoculated into 30 mL sterile starch casein broth and incubated for 8 d at 28 °C. After incubation, the culture was centrifuged at 5 000 r/min for 20 min. Using agar well diffusion method, the cell-free supernatant was assayed for antimicrobial activity against the following microorganisms: methicillin resistant *S. aureus*, *S. aureus* ATCC 29213, *Escherichia coli* ATCC 29522 (*E. coli*), *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* (*C. albicans*), *Enterococcus faecalis* ATCC 29212. Coagulase-negative staphylococci isolated from HIV patients were also used (*Staphylococcus warneri*, *Staphylococcus xylosus* and *Staphylococcus epidermidis*). Sterile Mueller–Hinton and Sabouraud dextrose agar plates were seeded with test bacteria and yeast respectively and incubated at 37 °C for 24 h while those seeded with the yeast were incubated at 37 °C for 48 h[12].

2.5. Gas chromatography-mass spectrometer (GC-MS) analysis of crude extract

Extraction of secondary metabolites was carried out using the method of Mohkam *et al*. with modifications[13]. Twenty millilitre of cell-free crude extract was mixed with a combination of ethyl acetate/methanol (1:1) in a separating funnel and shaken vigorusly for 30 min and afterwards, was allowed to stand without any disturbance for 15 min. The lower aqueous phase was discarded and the organic phase was collected into a glass beaker and concentrated to 1 mL. A standard (pure) for the antibiotic combinations was first injected into the GC to set its equivalent peak area and rention time profiles of the individual antibiotics. Afterwards, 0.1 uL was injected in to GC 6890 series (Hewlett Packard) with specification (column size 0.25 mm×30 m, carrier gas nitrogen, flow rate 22 mL/min, injection temperature at 220 °C, acceleration and reflector temperature 100 °C/min, initial column temperature at 50 °C, holding time 2 min). The peak area of the standard antibiotics were compared to those of the test samples.

3. Results

Three isolates (ULS12, ULS13 and ULK3) suspected to be actinomycetes grew on the starch casein and Kuster's agar suplemented with cycloheximide. The mycelia of ULS12 colonies were white and turned grey with age and produced brown pigment in agar while that of ULS13 were white, powdery turning grey with age and produced yellow pigment. ULK3 colonies were faint green and powdery with no pigment in agar.

The result of the physicochemical characteristics of the suspected actinomycetes isolates was shown in Table 1. The organisms were non-sporulative and showed ability to utilize glucose, lactose, saccharose, maltose while none of the isolates were able to utilize urease, cellobiose, gelatine, mannose, melezitose, rhamnose. All isolates were however found to be catalase negative but able to hydrolyze starch.

The species-specific primers used confirmed the strains to be actinomycetes based on the amplification of the 640-bp stretch of the 16S rRNA gene. The results of the sequences analysed using basic local alignment search tool showed ULS12 having 99% similarity

Table 1

Physicochemical characteristics of the actinomycetes isolates.

Isolate	IND	URE	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GEL	ESC	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE	CAT	SPO	GRM	STA	CAS
ULS12	-	-	+	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+
ULS13	-	-	+	-	+	+	+	+/-	+	+/-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-
ULK3	+	-	+	+	+	+	+	+	+	-	-	+	-	-	-	-	+	+	-	+	-	-	+	+	+

IND: Indole; URE: Urease; GLU: Glucose; MAN: Mannitol; LAC: Lactose; SAC: Saccharose; MAL: Maltose; SAL: Salicin; XYL: Xylose; ARA: Arabinose; GEL: Gelatin; ESC: Esculin; GLY: Glycerol; CEL: Cellobiose; MNE: Mannose; MLZ: Melezitose; RAF: Raffinose; SOR: Sorbitol; RHA: Rhamnose; TRE: Trehalose; CAT: Catalase; SPO: Spores; GRA: Gramreaction; STA: Starch Hydrolysis; CAS: Casein Hydrolysis.

to *Streptomyces albus* J1074 but ULK3 showed a higher similarity (100%) to *Streptomyces albus* J1074 while ULS13 showed 100% similarity to *Streptomyces fulvissimus* DSM 40593.

The crude extracts from the isolates were screened for antimicrobial activity against pathogenic microorganisms and it was observed that isolate ULS12 and ULS13 displayed significant inhibitory activity against bacterial isolates such as *E. coli* ATCC 29522 (Figure 1) while ULS13 showed the highest antifungal activity against *C. albicans* (Figure 2). ULK3 showed activity against *C. albicans* only as shown in Table 2.

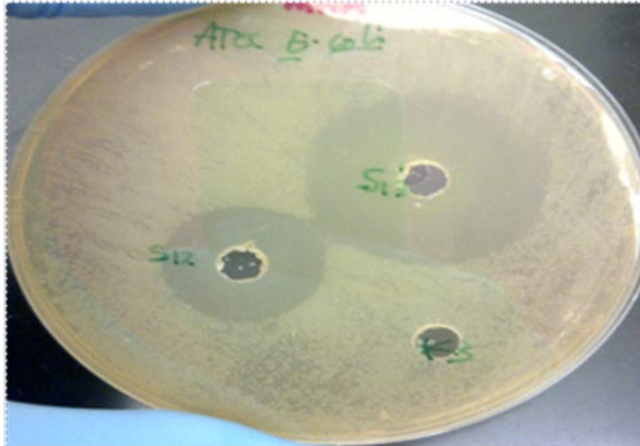

Figure 1. Antibacterial activity of actinomycete ULS13 and ULS12 against *E. coli* ATCC 29522.

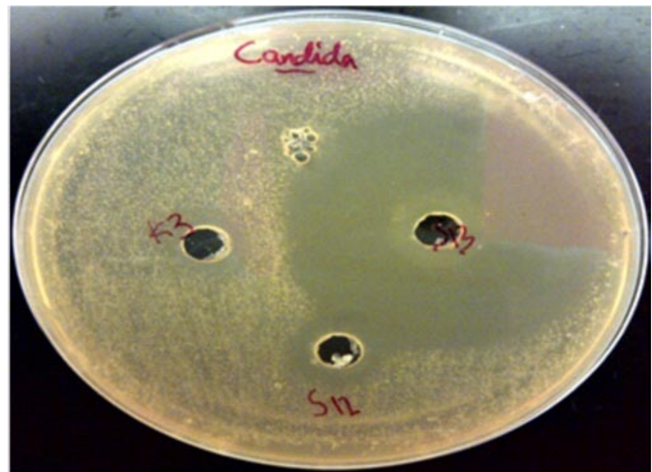

Figure 2. Antifungal activity of actinomycete ULS12 against *C. albicans*.

Figures 3, 4 and 5 show the result of the GC-MS analysis of the crude extracts. The ethyl acetate/methanol extracts of the isolates identified 19 different types of antibiotics with varying number of peak values at different time intervals. Peaks indicating the presence of rifamycin B&SV, erythromycin, tetracenomycin, kanamycin, nystatin, oxytetracycline, tylosin, marinomycins, asukamycin, chloramphenicol, glaciapyrroles, cycloheximide, amythiamicins, streptomycin, ipomicin, capuramycin, avilamycin, tubelactomicin and resistoflavin were detected in the crude extracts. All isolates were found to produce

Table 2

Antimicrobial activities of crude cell-free extract against pathogenic microorganisms.

Isolates	Zone of inhibition (mm)								
	S. warneri	Methicillin-resistant *S. aureus*	*S. xylosus*	*S. epidermidis*	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 29522	*E. faecalis*	*S. aureus* ATCC 29213	*C. albicans*
ULS12	12	29	18	12	9	15	26	36	3
ULS13	13	20	8	14	10	18	27	29	18
ULK3	-	-	-	-	-	-	-	-	3

S. warneri: *Staphylococcus warneri*; *S. xylosus*: *Staphylococcus xylosus*; *S. epidermidis*: *Staphylococcus epidermidis*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *E. faecalis*: *Enterococcus faecalis*.

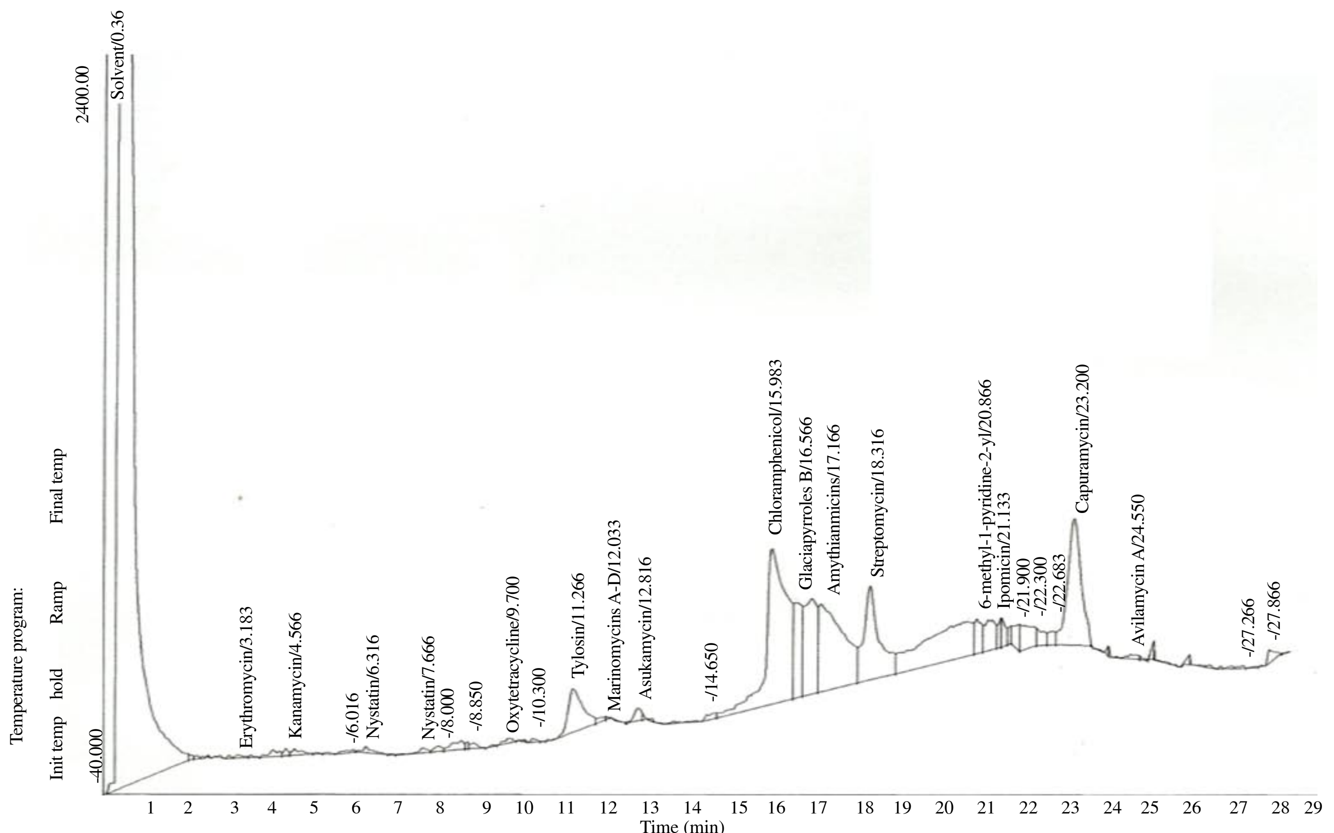

Figure 3. Detection of antibiotics present in the crude extract of ULK3.

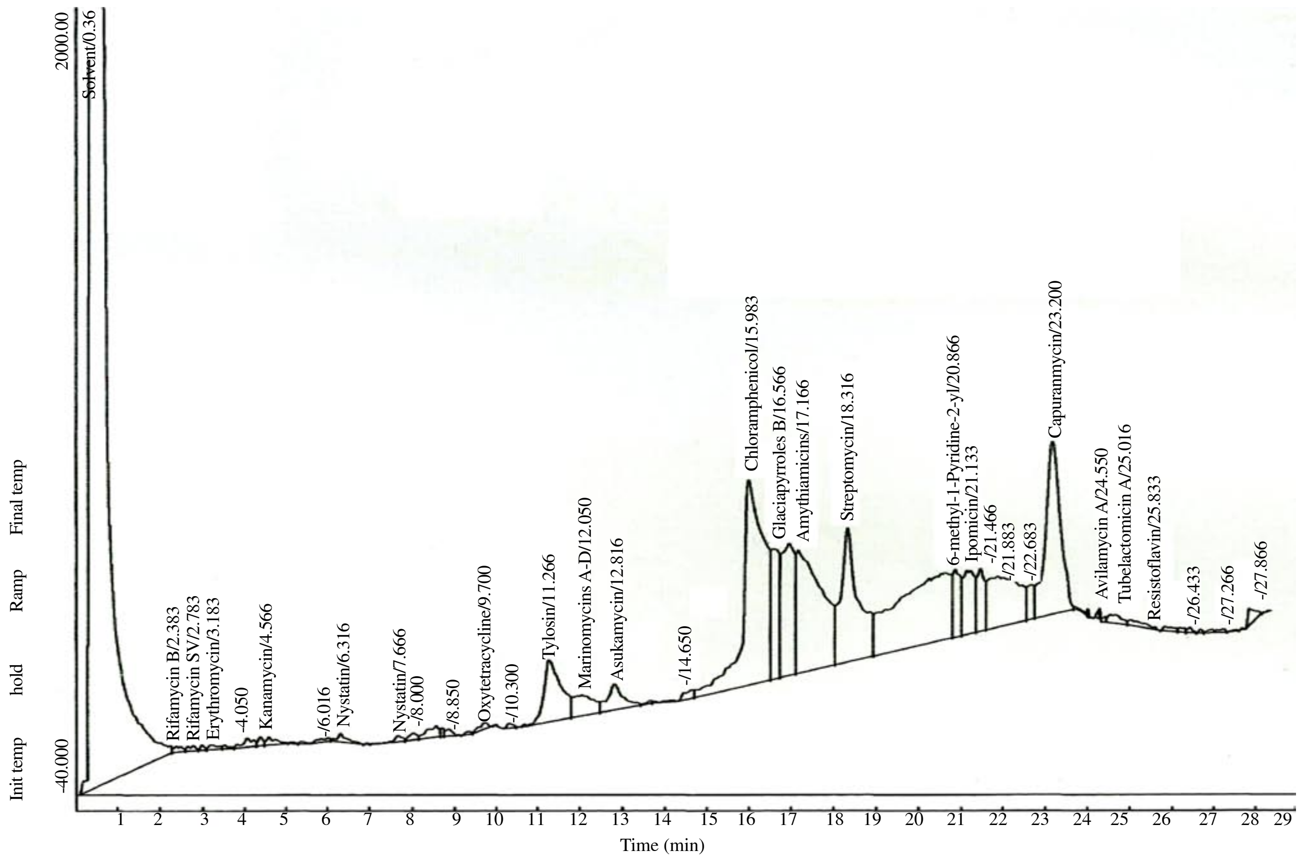

Figure 4. Detection of antibiotics present in the crude extract of ULS13.

Figure 5. Detection of antibiotics present in the crude extract of ULS12.

ipomicin, capuramycin and tylosin.

Table 3
Peak profile of methanolic extract of all the strains.

Component		Retention (min)	Area	Height
ULK3	Solvent	0.366	114173.2340	5614.853
	Erthromycin	3.183	103.4940	8.181
	Kanamycin	4.566	337.6520	21.000
	Nystatin	6.316	315.8570	21.748
	Nystatin	7.666	198.4545	15.623
	Oxytetracycline	9.700	426.1710	23.717
	Tylosin	11.266	4388.5030	156.194
	MarinomycinsA-D	12.050	1803.0180	53.879
	Asukamycin	12.816	1716.5540	66.267
	Chloramphenicol	15.983	17873.1570	510.598
	Glaciapyrroles B	16.566	4439.8410	322.791
	Cycloheximide	16.950	6835.5000	325.048
	Amythiamicins	17.166	12408.2700	302.626
	Streptomycin	18.316	9022.6910	328.172
	6-methyl-1-pyridine	20.866	1880.8620	156.204
	Ipomicin	21.133	2843.4960	147.240
	Capuramycin	23.200	9664.5160	413.783
	Avilamycin A	24.550	288.7175	14.368
	Resistoflavin	25.833	104.6645	17.477
ULS12	Solvent	0.366	116562.5600	5619.350
	Rifamycin B	2.383	329.3100	31.366
	Rifamycin SV	2.783	294.0350	20.315
	Erthromycin	3.183	334.3105	18.944
	Tetracenomycin D	3.533	180.4750	15.862
	Kanamycin	4.566	519.7390	26.523
	Nystatin	6.316	411.0310	24.627
	Nystatin	7.666	196.4545	15.623
	Oxytetracycline	9.700	251.0870	16.839
	Tylosin	11.266	4431.8630	156.872
	MarinomycinsA-D	12.050	1889.2820	55.782
	Asukamycin	12.816	1938.5405	69.851
	Chloramphenicol	15.983	19602.8780	530.549
	Glaciapyrroles B	16.566	4811.5830	348.823
	Cycloheximide	16.950	7488.4600	355.075
	Amythiamicins	17.166	14449.7780	334.912
	Streptomycin	18.316	11456.6630	372.445
	6-methyl-1-pyridine	20.866	2807.6060	227.058
	Ipomicin	21.133	4402.5675	220.873
	Capuramycin	23.200	16876.6710	508.959
	Avilamycin A	24.550	1749.3930	53.683
	Tubelactomicin A	25.016	1042.7625	32.571
	Resistoflavin	25.833	314.9500	16.636
	Total		212341.9995	
ULS13	Solvent	0.366	115261.3370	5616.928
	Rifamycin B	2.383	214.6795	15.566
	Rifamycin SV	2.783	164.6330	15.156
	Erthromycin	3.183	179.9920	12.151
	Kanamycin	4.566	337.4650	20.995
	Nystatin	6.316	315.7420	21.745
	Nystatin	7.666	196.4545	15.623
	Oxytetracycline	9.700	235.9560	15.677
	Tylosin	11.266	4366.3430	155.879
	Marinomycins A-D	12.050	1768.9060	53.101
	Asukamycin	12.816	1641.6275	65.036
	Chloramphenicol	15.983	18180.8095	514.276
	Glaciapyrroles B	16.566	4510.7090	327.744
	Cycloheximide	16.950	6961.2960	330.839
	Amythiamicins	17.166	12807.9980	308.891
	Streptomycin	18.316	9506.1835	336.948
	6-methyl-1-pyridine	20.866	2068.5755	170.553
	Ipomicin	21.133	3159.7245	162.171
	Capuramycin	23.200	10707.7495	162.171
	Avilamycin A	24.550	465.2460	21.101
	Tubelactomicin A	25.016	403.4450	12.896
	Resistoflavin	25.833	195.6125	11.077
	Total		193652.4845	

The GC-MS result of the ethyl acetate/methanol extracts of the isolates showed varying number of peak values with different time intervals. The chromatograph (Figures 3, 4 and 5) showed total of 44, 37, 34 peaks for crude extracts of isolates ULS12, ULS13, ULK3 respectively. The number of identified peaks were 22, 20, 17 peaks for extracts of isolates ULS12, ULS13, ULK3 respectively. The identified antibiotics were extracts from ULS12 and ULS13 with the exception of the extract from isolate ULK3 were found to contain rifamycin B & SV as well as tubelactomicin and resistoflavin. Tetracenomycin was also found in ULS12. Therefore in all, the extracts from isolates ULS12 and ULS13 were found to have the highest number of both identified and unidentified peaks.

4. Discussion

The reduction in the frequency of novel drug discovery from well-explored terrestial environment has given rise to exploration of the marine environment, which has been largely overlooked, for novel broad spectrum antibiotics. An attempt was therefore made in this study to isolate actinomycetes from sediment of Lagos Lagoon to evaluate their antimicrobial potential.

Molecular approaches such as PCR amplification of genes for identification have been used over many decades because identification of microorganisms based on biochemical characteristics has been found to be inadequate. All the isolates showed positive results as amplification using the species specific primers confirmed all the isolates to be actinobacteria[10]. The blast analysis of the sequences identified all the isolates as *Streptomyces* sp.

The isolate ULK3 exhibited only antifungal properties and no antibacterial activities were displayed by this isolate. This is similar to the report of Yang *et al*.[14] and Oskay *et al*.[15].

The extracts from ULS12 and ULS13 were found to contain rifamycin B & SV as well as tubelactomicin A. Rifamycins are clinically important antibacterial agents active against Gram-positive bacteria[16]. Tubelactomicin A has also been reported to show activity against drug-resistant pathogenic strains[17]. The presence of these two antibiotics in ULS12 and ULS13 extracts may explain the high antimicrobial activity recorded against methicillin–resistant *S. aureus* and all the other pathogenic bacterial strains screened. This observation is in line with previous study carried out by Singh *et al*. who isolated an actinomycete which displayed antibacterial activity against methicillin-resistant *S. aureus*[7]. The extract from isolate ULS12 was found to have the highest number of unidentified peaks followed by those of ULS13. This development could account for the high broad spectrum antimicrobial activities exhibited by these isolates because of the presence of unidentified antibiotics and could be an indication of the presence of some novel antibiotics since this would be the first report of antibiotic-producing actinomycetes from Lagos lagoon enviroment.

The Lagos lagoon in the West African Coast is an unexplored area for novel drug discovery. Therefore, the findings from this study highlights the potentials of the marine actinomycetes of the Lagos

marine environment as a source of novel antimicrobial compounds which could contribute to current efforts aimed to control drug-resistant pathogens.

Acknowledgement

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Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

Antimicrobial secondary metabolites of *Streptomyces* sp., isolated from Lagos lagoon soil were screened for antimicrobial activity against antimicrobial resistance strains. Antimicrobial resistance is a global threat and this study proposed antimicrobial activity against methicillin resistant *S. aureus*, which is of prime significance.

Research frontiers

The present research article proposes antimicrobial efficacy of *Streptomyces* sp. ULS12 and ULS13 against methicillin resistant *S. aureus*. There identified metabolites could pose as a treatment strategy against methicillin resistant *S. aureus*.

Related reports

The observation are in line with previous study carried out by Singh *et al.* who isolated an actinomycete which displayed antibacterial activity against methicillin-resistant *S. aureus*[7].

Innovations and breakthroughs

The extracts from ULS12 and ULS13 were found to contain rifamycin B&SV as well as tubelactomicin A, which has already been reported in previous studies. Tests and methods used in study are well established.

Applications

The extracts from ULS12 and ULS13 were found to contain rifamycin B&SV as well as tubelactomicin A. The major findings from this study highlight the potentials of the marine actinomycetes of the Lagos marine environment as a source of novel antimicrobial compounds. These peptides have potential to be explored against antimicrobial resistance strains with application of recombinant DNA technology and bulk production.

Peer review

This is a valuable research work in which authors have demonstrated the antimicrobial efficacy of actinomycetes isolated from lagos marine environment. GC-MS studies identified rifamycin as well as tubelactomicin as the inhibitory peptides.

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