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Original Research Article

Isolation and characterization of terrestrial *Streptomyces* strains with antimicrobial and anti-ulcer activities

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Abstract

Purpose: *To determine the antimicrobial, anti-ulcer and cytotoxic activities of secondary metabolites isolated from Streptomyces spp in soil samples obtained from Anyigba, Kogi State, Nigeria.*
Methods: *The cytotoxic activity of Streptomyces secondary metabolites (concentration range: 62.5 – 1000 mg/mL) was assessed using brine shrimp lethality assay on different concentrations (62.5, 125, 250, 500, 1000 mg/mL) of the secondary metabolites or the reference $K_2Cr_2O_7$. The anti-ulcer activity was evaluated using aspirin and ethanol models of ulcers in albino rats. Five groups of animals viz three pretreated groups with the extracts at two different oral concentrations of 100 and 200 mg/kg, one pretreated group with omeprazole (30 mg/kg) as standard and another group with oral 2 mL/kg normal saline (control). Antibacterial and antifungal activities were investigated using the microdilution method.*
Results: *Cytotoxicity test showed that extract concentrations of 12.5 to 62.5 mg/mL exerted mild toxicity compared to control. For antiulcer activity, animals in group 1 showed mucosal infiltration of leucocytes, massive exfoliation of epithelial cells and cellular hemorrhage while group 2 had mild tissue erosion and small ulcer. In groups 3 to 5, extract at 200 mg/kg showed excellent cytoprotection and proficient curative capacity in contrast to the 100 mg/kg, without significant side effects while the standard group showed some side effects and a significant reduction in the mucus cells ($p < 0.05$). The secondary metabolites inhibited ulcer-implicated organisms (Helicobacter pylori, Escherichia coli, Campylobacter spp and Streptococcus spp), exerted curative effect and protected the rats from ulcers.*
Conclusion: *Secondary metabolites isolated from Streptomyces spp were not toxic to epithelial tissue and exhibit antimicrobial and anti-ulcer activities and thus has potential as an important source of drugs against peptic ulcers.*

Keywords: *Anti-ulcer, Secondary metabolites, Cytotoxic Activity, Streptomyces sp*

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INTRODUCTION

Over the recent decades, there has been a growing interest in the search for novel, bioactive, sustainable and broad-spectrum antimicrobial agents from various sources including microbes for use against most life-threatening infectious diseases caused by microbes [1-3]. This is because the rapidly evolving multidrug resistance by pathogenic microorganisms has compromised the efficacy of most existing antibiotics [2]. Globally, natural products have been recognized as one of the principal sources of bioactive secondary

metabolites with novel structural and functional diversity for pharmacological therapy and prevention of most human diseases [4]. Of the microbial natural sources, actinomycetes usually constitute a significant potential microbial source of economically and industrially important bioactive secondary metabolites [2,5]. The genus *Streptomyces* represents a significant proportion of the total actinomycetes population and is generally recognized as a proficient producer of novel bioactive molecules in the natural soil habitat [5]. Worldwide, the isolation, identification and characterization of new strains of *Streptomyces* have continued to be a growing field of discovery [3]. Even though soils have been screened in other ecological settings for innovative biomolecules with numerous pharmaceutical, agricultural, industrial and therapeutic applications, the information from those sampled areas may not be generalizable as only a small portion of the earth's surface and actinomycetes taxa are studied [2].

Streptomyces have been isolated from many terrestrial habitats across the globe; their relative abundance and ability to produce bioactive metabolites are largely dependent on their habitat, study location and environmental factors [3]. The unique assets of Nigerian biodiversity can serve as a treasure for the search for novel isolates.

Thus, the current study was designed to screen soil samples from five different unexplored agroecological niches in the Anyigba area of Kogi State, to isolate, identify and characterize *Streptomyces* spp. Furthermore, the study assessed the antibacterial and antifungal activities of the extract as well as the anti-ulcer activity and cytotoxicity of secondary metabolites from the soil-based *Streptomyces* in Anyigba, Kogi State, Nigeria.

EXPERIMENTAL

Study area

This study was carried out in Anyigba, situated in Dekina Local Government Area of Kogi State, Nigeria. The city is located on latitude 7^{o} 15^{1} – 7^{o} 29^{1} North of the Equator and Longitude 7^{o} 11^{1} – 7^{o} 32^{1} East of the Greenwich Meridian. The climate of Anyigba corresponds with that of the tropical hinterland and the Guinea savanna [6] and it is characteristically hot and dry, with the mean annual temperature and rainfall at 25 ^{o}C and 1600 mm, respectively.

Sample collection and preparation

Several diverse microbial habitats in different areas within Anyigba, Kogi State, Nigeria were selected for isolation of *Streptomyces* using the method of Bello and Nwankwo [7]. These habitats include cassava farmlands, cashew farmlands, grasslands, yam farmlands and general refuse dumps that were located between 3 – 5 km from each other within Anyigba. Approximately 20 g of soil samples were obtained from a depth of 3 – 4 cm of the soil surface using a hoe. Samples were placed in polythene bags, tightly closed and labeled according to the source of excavation. They were transported to the Microbiology Laboratory at the Kogi State University and subsequently allowed to dry at room temperature (30 ± 2 ^{o}C) for a week [1,7].

Isolation and preliminary identification of *Streptomyces spp*

Twenty grams (20 g) of each soil sample was dissolved in 180 mL of distilled water, rocked gently and serially diluted to a dilution factor of 10^{-5} as described elsewhere [8]. The different sample dilutions were inoculated using the method and media employed by Kaiser [8]. Simple stain and gram stain were performed on the *Streptomyces* isolates as described by Cheesbrough [9] while the microscopic characteristics such as spore and Mycelia arrangements were carefully observed. Biochemical tests such as catalase, Urea hydrolysis, citrate utilization, carbohydrate fermentation and starch hydrolysis tests were performed as described by Kaiser [8].

Extraction of DNA from Isolates

DNA was extracted from 200 µL of each of the pure isolates using the Macherey-Nagel viral DNA/RNA Mini Kit (Thermofisher Scientific, UK) following the manufacturer's instructions. The resulting DNA eluted in nuclease-free water was stored at -80 ^{o}C before use.

Molecular detection and characterization of *Streptomyces* isolates through sequencing

The template DNA was amplified using a set of published primers (forward: 5′AGAGTTTGATCCTGGCTCAG-3′; reverse primer: 5′-CCGTACTCCCCAGGCGGGG-3′) in an Eppendorf PCR thermal cycler [3]. The PCR amplification comprised 30 cycles of denaturation at 91 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 ^{o}C for 1 min and final extension at 72 °C for 5 mins. The resulting amplicons

were analyzed using 1.5 % agarose gel electrophoresis. Electrophoresis was carried out at 80 V and 400 A for 60 mins using 100 bp DNA ladder. The amplicons were purified using a QIAGEN quick gel extraction kit (QIAGEN, Maryland, USA) and sequenced by First BASE Laboratories, Malaysia using an automated DNA sequencer (Applied Biosystems ABI-3730-XL, USA). Forward and reverse primers were used to sequence the target gene. Sequences were compared with other pools of organisms using the NCBI BLAST tool for ancestral genomic similarity pairing. A phylogenetic tree depicting the evolutionary relatedness of terrestrial *Streptomyces* strains isolated in Anyigba with global reference strains was inferred using the MEGA 7 software and the neighbor-joining algorithm.

Screening for secondary metabolites

The pure culture of *Streptomyces* isolates, with the end of diffusible pigmentation in the substrate mycelia, was sub-cultured in an already prepared Czapadox broth for 10 – 14 days in an orbital shaker [10]. The culture was centrifuged at 6,000 revolutions per minute (rpm) for 5 mins and it was filtered using a sterile filter paper. The filtrate was further centrifuged at 1000 rpm for 30 minutes. Afterwards, it was filtered using a sterile filter paper, and the broth culture was then concentrated into powdered form using methanol in a rotatory evaporator [11]. The evaluation of secondary metabolite production was done using the Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

Antimicrobial spectrum of isolates

The antimicrobial activity of the isolates (concentration range: 12.5 – 62.5 mg/mL; control: 12.5 mg/mL) was tested according to the method of Kaiser [8], using ulcer-implicated test organisms (*Helicobacter pylori, Escherichia coli, Campylobacter spp and Streptococcus spp*). In the primary screening, the test organism was streaked perpendicularly against the *Streptomyces* isolate (five days old culture) on nutrient agar and incubated for a day at room temperature (30 ^{o}C) according to the method of Kizito and Nwankwo [7]. The antimicrobial activity of the Streptomyces isolates was measured to the nearest millimeters and recorded appropriately.

Brine shrimp cytotoxicity assay

A brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of *Streptomyces* secondary metabolites. A fixed volume (100 µL) of each concentration (62.5, 125, 250, 500, 1000 mg/mL) of the secondary metabolites or the reference, potassium dichromate ($K_2Cr_2O_7$), was added to the vials containing the hatched brine shrimp (*Artemia salina*) larvae as described by Meyer *et al* [12] and modified by Arogba [13].

Antiulcer activity of the S*treptomyces* secondary metabolite extract

Two models of ulcer were used namely the aspirin and alcohol-induced models of ulcer. In the aspirin-induced ulcer model, the method of Herbert *et al* [14] was employed. White albino rats (Wistar strain) of either sex, aged between 2 – 3 months and weighing between 78.38 – 177.30 g, were used. The animals were raised under favourable growth conditions (temperature: 30 ± 4 °C; *relative humidity: 60* %) in the animal house. The diet was a mixture of wheat flour (18 %), corn meal (38 %), fish meal (18 %), soybean meal (12 %), wheat flour (10 %), palm kernel (3 %), common household salt (0.6 %) and vitamin complex (0.4 %). Adult albino rats were randomized into 5 groups of 10 animals each. Ulceration was induced via oral administration of aspirin at a dose of 200 mg/kg and omeprazole administration was after 5 hrs. Groups 1 and 2 served as normal and standard control groups and were orally administered 2 mL/kg of normal saline and Omeprazole (30 mg/kg), respectively. Group 3 was given the chloroform extract of *Streptomyces* secondary metabolites at two different concentrations of 100 and 200 mg/kg body weight *(Rats weighing between 78.38 – 177.30 g were used for the screening as indicated above. Animals in the group with small body mass (≤100 g) received 100 mg/kg of the extract while the ones with larger body size (>100 g) received 200 mg/kg)*. Similarly, Groups 4 and 5 received the ethyl acetate and ethanol extracts of *Streptomyces* secondary metabolites, respectively [15,16]. After 8 days of daily treatment, the animals were euthanized using anesthetic ether, their stomachs were opened and the ulcer index was calculated [15]. In the alcohol-induced gastric ulcer model, the albino rats were randomized into another 5 groups of 10 rats and fasted for 24 h but with access to water. Groups 1 and 2 served as normal and standard control groups and were orally administered 2 mL/kg of normal saline and Omeprazole (30 mg/kg), respectively. Group 3 received chloroform extract, Group 4 received ethyl acetate extract while Group 5 was given the ethanol extract at oral concentration of 100 or 200 mg/kg, depending on body weight. One hour later, 1 mL of 80 % ethanol was administrated orally to each animal in all groups [16]. Animals were sacrificed by cervical dislocation one hour

after ethanol administration, the stomachs were isolated and cut open along the greater curvature and pinned on a soft board. The length of each gastric lesion was measured and the lesion index was expressed as the sum of the length of the entire lesion in mm.

In the calculation of the ulcer index, it's imperative to consider one choice of method since this could affect the relevance of the results obtained [17]. The ulcer index (UI) was calculated using the Kulkarni method in Eq 1 [17].

UI = 10x …….. (1)

where x = total mucosal area divided by total ulcerated area.

However, in the aspirin ulcer model, the protection % was calculated using the protective approach using Eq 2.

Preventive (%) = ($UI_{control}$ – $UI_{pretreated}$/$UI_{control}$)100 …… (2)

In the alcohol model, using the curative approach, the curative percentage was calculated using Eq 3.

Curative (%) = ($UI_{control}$ – $UI_{treated}$/$UI_{control}$)100 …… (3)

Statistical analysis

Descriptive data were presented as simple summaries expressed as mean ± standard deviation (SD). Data generated in the current study were analyzed using SPSS version 16 for Windows. Analysis of variance (ANOVA) was used to ascertain the differences between groups and $p < 0.05$ was set as the level of statistical significance.

RESULTS

Thirteen *Streptomyces* strains were isolated from soil samples obtained from agro-ecologically diverse habitats located at PAAU farm, in Anyigba (Table 1). The soil samples (designated A and B) obtained from Cashew farmland, Oganaji river (riverbank and creek), and Corn/Cassava farmland in PAAU farm had close fairly acidic pH (5.4 – 5.8) against the samples from the refuse dump in PAAU behind Ocheja Males Hostel, which had an alkaline pH ($p < 0.05$; Table 1). The strains were identified based on features which included the presence of a discrete, velvety, chalky and powdery aerial mycelium. Table 2 depicts the colonial characteristics of the isolates while Gram's reaction and biochemical characteristics are shown in Table 3. Two of the *Streptomyces* strains identified in the study (KSU.003 and KSU.004) demonstrated 100 % nucleotide similarity with reference *Streptomyces griseus* from the Genbank and are clustered together on the separate main phylogenetic tree. The field strains designated KSU.001 and KSU.002 had 99.88 % sequence identity with *Streptomyces anulatus* strain NBC (Figure 1).

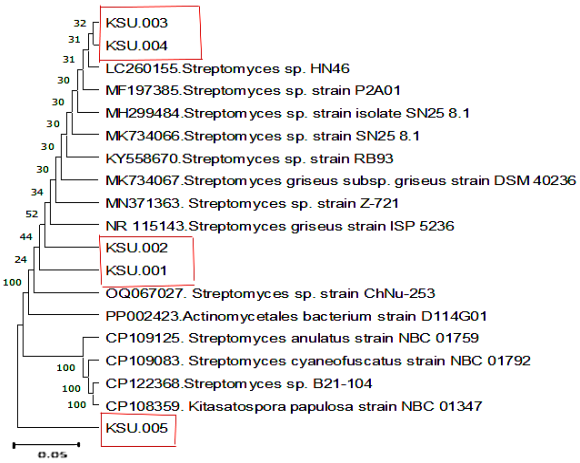


Figure 1: Phylogenetic tree showing the evolutionary relatedness of terrestrial *Streptomyces* strains isolated in Anyigba with global reference strains, based on their 16S rRNA gene sequences

The phylogenetic tree was inferred using the neighbor joining method and the bootstrap values were calculated based on 1000 replicates. The scale bar indicates a 0.05 nucleotide substitution position. The presence and percentage composition of the major secondary metabolites obtained via gas chromatography-

Table 1: Result of soil pH and sample collection site

Site of collection	pH	
	Sample A	***Sample B***
Cashew farmland	5.5	5.6
Oganaji river (riverbank and creek)	5.4	5.4
Refuse dump in PAAU (behind Ocheja Boys hostel)	8.1	8.2
Corn/Cassava farmland in PAAU farm	5.7	5.8

Table 2: Colonial morphology and pigmentation of isolates

Sample location site	Appearance	Elevation	Aerial pigment	Edge	Substrate pigmentation	Spore arrangement	Visible diffusible pigment
CC1	Dry and smooth	Convex	White	Fuzzy	Cream	Straight	Brown
CC2	Dry and rough granules	Convex	Greyish Black	Irregular	Grey	Spiral	Yellow
DP1	Smooth and dry	Convex	White	Fuzzy	White	Flexibillis	Whitish pink
DP2	Dry and powdery	Flat	Chocolate Brown	Entire	Brown	Flexibillis	Brown
OG1	Dry and powdery	Raised	White	Entire	Cream	Straight	Milky
OG2	Dry and smooth	Flat	Grey	Irregular	Golden yellow	Straight	Pink
CW1	Dry and smooth	Convex	Cream	Entire	Cream	Spiral	Whitish Blue
CW2	Rough and dry	Convex	Chocolate	Irregular	Yellow	Flexibillis	Oxblood

Note: CC = Corn and Cassava, DP = Dumpsite, OG = Oganaji, CW = Cashew, (+) = Positive, (-) = Negative

Table 3: Gram reaction and characteristics of biochemical test

Name	G. Rxn	Citrate	Urease	Starch	Glucose	Sucrose	Galactose	Lactose	Fructose	Coagulase	Indole	Maltose	Catalase
C1	+	+	-	+	+	+	+	+	-	-	+	+	+
C2	+	+	+	+	+	+	-	+	+	+	+	+	+
D1	+	+	-	+	+	-	-	+	+	+	-	+	+
D2	+	+	+	-	+	+	-	+	+	+	+	+	+
OG1	+	+	+	+	+	+	+	+	+	+	+	+	+
OG2	+	+	+	+	+	-	+	+	+	+	+	-	+
CF1	+	+	+	+	+	+	+	+	+	+	+	+	+
CF2	+	+	+	+	+	+	+	+	+	+	+	+	+

mass spectrometry analysis included n-hexadecanoic acid, benzene, 1,1'-oxybis(4-phenoxy-), benzene sulfonanilide, nonanedioic acid, 8-octadecenoic acid, methyl stearate, methyl 20-methyl-heneicosanoate, e-14-hexadecenal, ethyl ester, phytyl palmitate, 2-butenedioic acid, 9-eicosene, heneicosyl heptafluorobutyrate, bis (2-ethylhexyl) phthalate and 1,2-benzenediol, 3,5-bis (1,1-dimet hylethyl; Table 4; Figure 2).

Of the *Streptomyces* isolates that were tested for antifungal activity, isolates from dumpsite (D1-D3) demonstrated the highest activity against the test organisms (range of inhibition: 16 – 32 mm; Table 5).

Similarly, the *Streptomyces* isolate from dumpsite (D1-D3), with a level of inhibition ranging from 13 – 27 mm, showed the highest activity against Gram-positive and Gram-negative bacteria ($p < 0.05$), while the isolates from Oganaji River generally had the lowest antibacterial activity (Table 6).

Isolates from dumpsite soil samples showed a remarkable broad-spectrum activity on all test organisms owing to the production of bioactive secondary metabolites ($p < 0.05$). The isolate D1 with the highest activity was further used for cytotoxicity and antiulcer assays. In the brine shrimp lethality bioassays, soil S*treptomyces spp* and the positive control, potassium dichromate, exhibited significant cytotoxic activities in a concentration-dependent manner (Table 7 and Table 8; $p < 0.05$).

In the negative control, deaths were only observed at 18 and 24 hours (Table 9). The rate of mortality of brine shrimps in comparison with the positive control ($K_2CR_2O_7$) was high, suggesting toxicity of the substance. In the first period (6 hours), there was no death at doses of 62.5 and 125 mg/mL, while death occurred at 250, 500 and 1000 mg/mL, respectively, ranging from 10 to 15 %. After 12 hours, the death rate increased from 35 to 60 %, a rate that is almost half of the brine shrimps. In the third period (18 hours), there was a higher mortality of about 60 to 85 %. Finally, after 24 hours, almost all the brine shrimps had died (percentage mortality: 90 to 100 %).

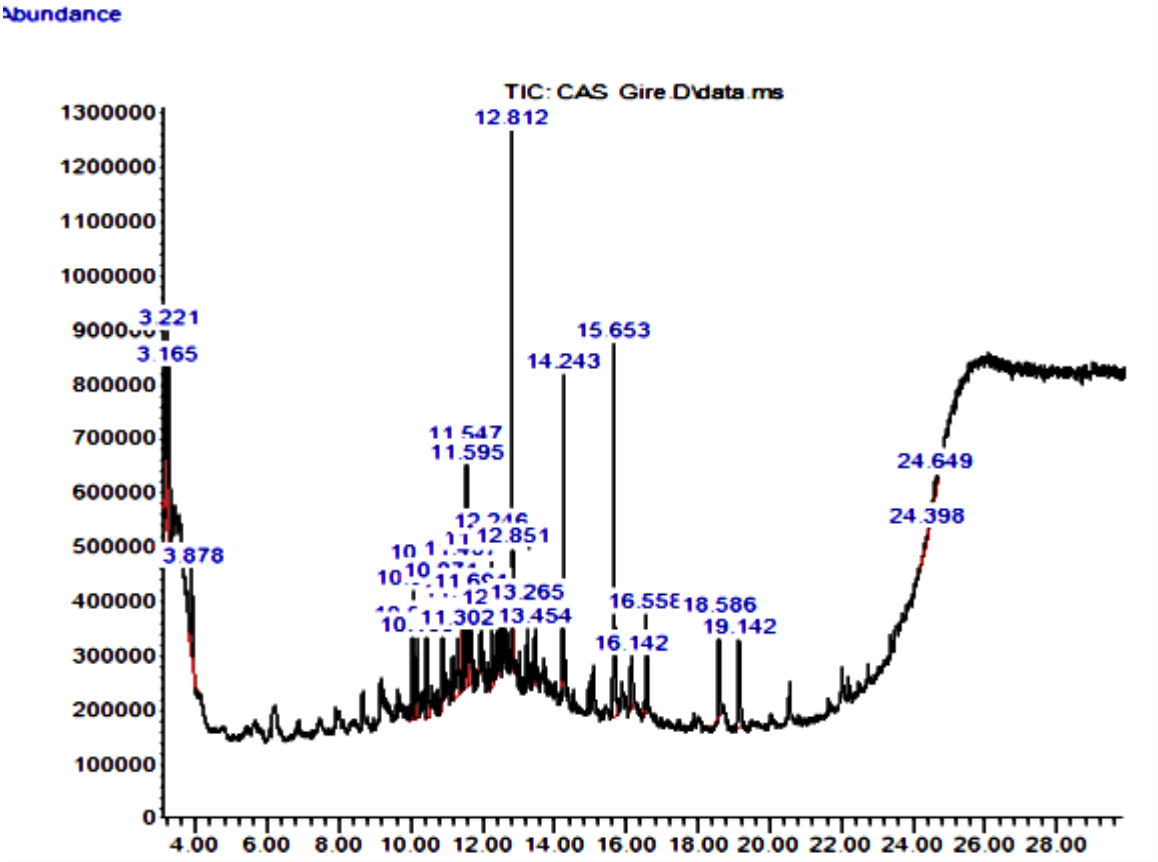


Figure 2: Total ion chromatogram (TIC)

Table 4: Quantitative analysis of the secondary metabolites of Streptomyces isolates extract using gas chromatography-mass spectrometry

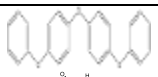
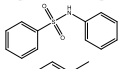
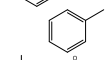
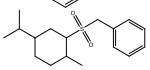
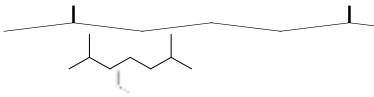
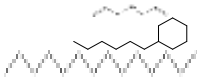
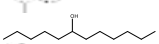
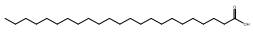
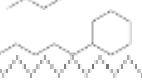
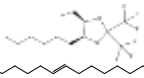
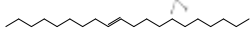
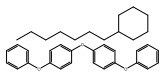
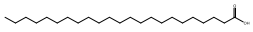
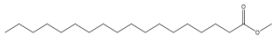
Compound	Retention Time	Percentage Composition	MF	MW	Structure
Benzene, 1,1'-oxybis (4-phenoxy-)	3.165	6.04	$C_{24}H_{18}O_3$	354.41	
Benzene sulfonanilide	3.221	10.35	$C_{12}H_{11}NO_2S$	233.29	
Toluene	3.878	3.45	C_7H_8	92.14	
((5-Isopropyl-2-methyl cyclohexyl) sulfonyl methyl) benzene	10.037	2.43	$C_{17}H_{26}O_2S$	294.45	
Heptane, 2,6-dimethyl-	10.081	3.04	C_9H_{20}	128.26	
Triallylsilane	10.168	2.08	$C_9H_{16}Si$	152.31	
Cyclohexane, hexyl-	10.442	3.14	$C_{12}H_{24}$	168.32	
Octadecane	10.871	2.56	$C_{18}H_{38}$	254.50	
3,3'-Bi-p-menthane	11.302	2.64	$C_{20}H_{38}$	278.52	
6-Dodecanol	11.407	2.38	$C_{12}H_{26}O$	186.34	
Cyclooctane, 1,2-diethyl-	11.434	1.43	$C_{12}H_{24}$	168.32	
2-Tridecene, (Z)-	11.547	5.18	$C_{13}H_{26}$	168.32	
Tricosanoic acid	11.595	6.14	$C_{23}H_{46}O_2$	354.62	
1,1,4-Trimethylcyclohexane	11.691	1.36	C_9H_{18}	126.24	
heptylcyclohexane	11.950	1.81	$C_{13}H_{26}$	182.35	
Nonadecane	12.246	2.60	$C_{19}H_{40}$	268.53	
1,3-Dioxolane, 4-ethyl-5-hexyl-2,2 -bis(trifluoromethyl)-, cis-	12.461	0.91	$C_{13}H_{20}F_6O_2$	322.29	
9-Eicosene, (E)-	12.812	7.56	$C_{20}H_{40}$	280.54	
Dotriacontane	12.851	1.92	$C_{32}H_{66}$	450.88	
heptylcyclohexane	13.265	0.92	$C_{13}H_{26}$	182.35	
Benzene, 1,1'-oxybis (4-phenoxy-)	13.454	0.84	$C_{24}H_{18}O_3$	354.41	
1-Octadecene	14.243	5.11	$C_{18}H_{36}$	252.49	
Hexadecanoic acid, methyl ester	15.653	10.46	$C_{17}H_{34}O_2$	270.46	
Tricosanoic acid	16.142	2.09	$C_{23}H_{46}O_2$	354.62	
1-Tricosene	16.558	3.06	$C_{23}H_{46}$	322.62	
10-Octadecenoic acid, methyl ester	18.586	3.83	$C_{19}H_{36}O_2$		
Methyl 20-methyl-heneicosanoate	19.142	4.44	$C_{23}H_{36}O_2$	354.62	
1,2-Bis(trimethylsilyl)benzene	24.398	0.63	$C_{12}H_{22}Si_2$	222.48	
4-(4-Hydroxyphenyl)-4-methyl-2-pentanone, TMS derivative	24.624	1.28	$C_{10}H_{14}O$		
1,2-Bis(trimethylsilyl)benzene	24.649	0.32	$C_{12}H_{22}Si_2$	222.48	

Table 10 and Table 11 show the weight of animals in each group used for aspirin and alcohol-induced ulcers, respectively. Generally, each group was found to experience weight loss following both treatments.

All the extracts exerted a higher level of protection against ulcers induced by aspirin (percentage protection: 72.5 – 89.1 %; Table 12) and alcohol (percentage protection: 72.5 – 89.1 %; Table 13) ($p < 0.05$), with correspondingly high pH than the control groups ($p < 0.05$).

Table 5: Antifungal activity of the S*treptomyces* isolates on the test organisms

Isolate	*Candida albicans*	*Aspegillus niger*	*Aspergillus flavus*	*Fusarium spp*
C1	0	16	0	12
C2	0	10	8	9
C3	5	10	10	12
D1	18	31	19	26
D2	16	28	17	28
D3	20	32	16	30
CF1	0	10	11	13
CF2	4	11	0	8
CF3	5	12	10	11
O1	8	11	6	10
O2	10	12	8	11
O3	0	12	5	15
O4	12	11	10	0

Note: C = Cashew Farm, O = Oganaji River, D = Refuse Dumpsite, CF = Cassava Farm; According to Kirby Bauer Standard: <_15 = Resistant (R); 16-20 = Moderate (M); >_21= Susceptible (S)

Table 6: Antibacterial activity of the *Streptomyces* isolates on the test organisms

Isolate	*Streptococcus spp*	*H. pylori*	*Campylobacter spp*	*E. coli*	*Salmonella spp*	*S. aureus*
C_1	4	11	13	22	12	10
C_2	6	10	14	21	11	13
C_3	20	12	11	8	20	11
D_1	25	23	22	24	22	15
D_2	25	24	26	27	23	16
D_3	24	16	25	20	20	13
CF_1	15	14	16	15	6	10
CF_2	9	11	20	12	7	9
CF_3	10	15	18	20	6	12
O_1	11	13	15	10	5	11
O_2	10	12	16	11	8	13
O_3	13	13	17	10	9	10

Note: C = Cashew Farm, O = Oganaji River, D = Refuse Dumpsite, CF = Cassava Farm

Table 7: Mean Percentage (%) lethality of brine shrimps at different intervals against secondary metabolite

Concentration (mg/mL)	6 h	12 h	18 h	24 h
62.5	10	13	50	80
125	17	23	53	83
250	17	27	56	93
500	13	33	60	96
1000	17	37	63	96

Table 8: Percentage (%) lethality of brine shrimp at different time intervals against positive control potassium dichromate

Concentration (mg/mL)	6 h	12 h	18 h	24 h
62.5	0	37	76	90
125	0	23	50	97
250	13	37	56	97
500	13	37	63	97
1000	10	37	83	97

Table 9: Percentage mortality of brine shrimp against negative control (artificial seawater) at various intervals (hour)

Concentration	Interval (h)	No. of Brine shrimp	Mean mortality	Percentage mortality (%)
1	6	40	0	0
2	12	40	0	0
3	18	40	1	10

Table 10: Weight (g) of animals in each group used for aspirin-induced ulcer

Group 1		Group 2		Group 3		Group 4		Group 5	
Before	***After***	***Before***	***After***	***Before***	***After***	***Before***	***After***	***Before***	***After***
145.58	121.13	116.64	97.6	137.62	120.13	78.38	50.19	172.64	108.18
135.24	104.16	108.93	92.09	116.86	98.7	88.92	71.05	168.28	109.07
150.57	100.08	116.94	94.26	140.89	111.18	95.2	74.19	177.3	112.15
119.42	97.07	98.54	90.17	138.68	107.17	86.32	60.27	175.35	121.61
145.55	113.27	101.98	95.17	129.61	120.18	88.91	70.11	152.41	110.07

Table 11: Weight (g) of animals in each chamber (alcohol-induced ulcer)

Group 1		Group 2		Group 3		Group 4		Group 5	
Before	***After***	***Before***	***After***	***Before***	***After***	***Before***	***After***	***Before***	***After***
113.5	100.01	120.64	110.7	137.62	120.13	78.38	50.19	172.64	108.18
148.06	132.09	108.93	92.09	116.86	98.70	88.92	71.05	168.28	109.07
150.57	100.08	119.94	97.26	140.89	111.18	95.20	74.19	177.30	112.15
119.42	97.07	98.54	90.17	138.68	107.17	86.32	60.27	175.35	121.61
145.55	113.27	101.98	95.17	129.61	120.18	88.91	70.11	152.41	110.07

Table 12: Ulcer index, protection and pH of aspirin-induced ulcerated rats

Group (mg/kg)	Ulcer index	Protection (mg/kg)	pH
Normal saline	4.2±0.01	25.0 ±1.43	2.5±0.11
OMZ (30)	3.2±0.05	35.0±1.44	2.0±0.31
Duplicate			
G-E 100	0.3±0.01	88.2±2.05	3.1±0.01
G-E 200	0.5±0.06	84.5±2.01	4.3±0.11
G-M 100	0.5±0.09	79.4±0.61	3.2±0.11
G-M 200	0.3±0.01	89.1±0.41	3.7±0.41
G-C 100	0.5±0.01	72.5±0.11	3.2±0.11
G-C 200	0.3±0.01	86.5±0.21	3.9±0.01

Note: OMZ: Omeprazole; G-E: Ethyl acetate extract; G-M: Methanolic extract; G-C: Chloroform extract of secondary metabolite

Table 13: Ulcer index, protection and pH of alcohol-induced ulcerated rats

Group (mg/kg)	Ulcer Index	Protection (mg/kg)	pH
Normal saline	8.2±0.11	31.2±0.19	2.4±0.91
OMZ. (30)	0.2±0.01	36.1±2.11	2.1±0.22
Duplicate			
G-E 100	0.40±0.11	82.0±3.22	3.5±0.11
G-E 200	0.42±0.00	81.2±0.15	4.1±0.00
G-M 100	0.43±0.10	80.2±0.11	4.9±0.17
G-M 200	0.30±0.01	81.3±0.30	4.0±0.14
G-C 100	0.32±0.20	82.6±3.17	4.6±0.00
G-C 200	0.40±0.00	71.1±0.11	4.8±0.14

Note: OMZ: Omeprazole; G-E: Ethyl acetate extract; G-M: Methanolic extract; G-C: Chloroform extract of secondary metabolite

DISCUSSION

Streptomyces is a naturally occurring bacterium in soil and are well-known group of actinomycetes with the ability to produce the most important secondary metabolites including antibiotics [3]. In this study, *Streptomyces* was mostly found in soil near neutral pH, an observation that has been previously reported [7]. The plausible reason for this observation could be that pH towards acidity or alkalinity may not be in favor of the growth and activity of *streptomyces*. The characterization of the various isolates through sequencing analysis showed that the soil-based *Streptomyces* strains were extremely related to each other (99 – 100 % sequence identity) and to the global reference strains (98 – 100 % identity) identified in the Genbank. Furthermore, the isolates were phylogenetically related to *Streptomyces griseus, Streptomyces anulatus* and *Streptomyces cyaneofuscatus*, which have been identified as important producers of secondary metabolites [1, 5]. The high antifungal and antibacterial activities observed with the isolates highlight their potential usefulness in the generation of important pharmacological agents against the tested fungi

and various Gram-positive and Gram-negative bacteria.

The results of GC-MS analysis are notably significant as very relevant secondary metabolites including *n*-hexadecanoic acid, nonanedioic acid, 8-octadecenoic acid, phytyl palmitate, 2-butenedioic acid and ethyl 9, 12, 15-octadecatrienoate were detected. In a previous phytoscan analysis, these similar bioactive molecules derived from a medicinal plant were noted to exhibit pharmacological activities such as anti-inflammatory, antibacterial, antioxidant and anticancer activities [18]. In another study, hexadecanoic acid was shown to exert a strong antimicrobial and anti-inflammatory activity [19]. Thus, the presence of these bioactive molecules from soil-derived *Streptomyces* could be responsible for the observed antifungal, antibacterial and antiulcer activities in this study.

The brine shrimp lethality bioassay showed that soil S*treptomyces spp* secondary metabolites exhibited significant cytotoxic activities and the level of cytotoxicity was comparable with the report of Bastos *et al* who showed that the extract from the microorganism possesses a reasonable level of cellular toxicity [20]. In the first period of observation (+6 hours), the mortality of the organism occurred at each dose resulting in 10 to 17 % deaths. Since the shrimps were exposed to substances alien to them, the environment became new resulting in increased mortality. Other factors such as absence of food, temperature and pH may play an influencing role. In the second period (12 hours), the percentage mortality of the brine shrimps became uniform, suggesting that the percentage mortality increased with an increase in the dosage or concentration of secondary metabolites from the isolates. This finding corresponds with the report of Dancer [21] and could be due to the shrimp's ability to adapt or acclimatize to the toxic substances in the environment. The cell's level of acclimatization agrees with the report of Arogba, *et al* who suggested that the cell is capable of independent proliferation to attain a level of tolerance to foreign compounds which may likely be toxic [13]. After 18 hours, there was an increase in mortality (50 to 65 % death) and the high mortality could be because of several interrelated factors such as cellular cytotoxicity and/or accumulation of toxic substances from the external environment.

The finding of this study disagrees with the study of Yakubu and Afolayan [22] who reported that at higher intervals there is a low degree of mortality and hence less cytotoxicity. At this period, the organism can no longer withstand the toxic substances. In the fourth period (24 hours), there was a higher death rate of about 80 to 96 %. The results of the positive control and the variation in the level of mortality are probably due to their toxic nature. It also justified the use of $K_2Cr_2O_7$ as a positive cytocellular toxic substance in the pharmacological determination of potential toxic properties of a bioactive compound. In the negative control (artificial seawater), during the first period (6 hours) there was no death observed. At this period, the environment is favorable for their survival, in which case, no toxic substance was added. In the second period (12 hours), almost 40 % of the brine shrimp had died, likely due to starvation and other environmental factors like temperature, pH and stress. In the third period (18 hours), 55 % died, which is nearly half of the brine shrimp. In the final period (24 hours), the percentage of mortality had increased to 86 %.

In the present study, the significant increase in ulcer index and gastric volume following oral administration of aspirin in the ulcerated rats may be attributed to either free radical formation or inhibition of prostaglandin synthesis. These findings agree with the report of Zlabek and Anderson where aspirin was reported to have caused alterations in gastric secretion of rats [23]. Conversely, pretreatment with the three extracts significantly reduced these parameters. In fact, the effects noticed for pH compared favorably with the negative control and standard drugs used in this study and were indeed suggestive of their possible gastroprotective attributes. In this study, the increased pepsin activity coupled with a decrease in mucus secretion in the alcohol and aspirin-ulcerated rats may indicate altered hydrophobicity and reduced protective ability of mucosal membrane against hemorrhagic erosion, thus, resulting in tissue damage. Pretreatment with the extract, however, facilitates the ulcer healing process, which is associated with decreased pepsin activity and elevated mucus levels in the gastric mucosa. This in turn, may encourage speedy wound healing of ulcerated areas of the mucosal epithelial and shield the gastrointestinal membrane, thus abrogating the catastrophic influence of aspirin and alcohol in ulcerated rats [24]. This is indicative of enhanced mucus secretion potential of the extract and suggestive of their significant role in the ulcer healing process.

In the aspirin-induced ulcer model, Group 1 (control) which received normal saline, presented with hemorrhage, infiltration of inflammatory cells, mass exfoliation of the epithelial cells and leucocyte infiltration of submucosal layers as well

as sore or blister in the mouth of the animals and bloody urine. The group treated with Omeprazole had mild ulceration and the presence of inflammatory cells, blood-stained urine and blisters in the mouth. The extract showed a high protective ability and reduced mouth blisters. On the other hand, the extract (groups 3 to 5) showed a curative ability where healing lesions were seen with an indication of the absence of hemorrhage in the alcohol-induced ulcer model. The control group showed large (extensive) lesions and hemorrhage, inflammatory cell and leucocyte infiltration of the submucosal, layer and mass exfoliation of the epithelial layer. The Omeprazole-treated group showed the presence of mild lesions, erosion and inflammatory cells but not as prominent as the control group.

Overall, the normal saline could not protect against or cure the animal model from the ulcerogen. Omeprazole could protect and treat the ulcer, but experimental animals showed some considerable side effects like blood-stained blister on the tongue and reduced mucus membrane of the lungs and stomach. Treated animals in group 1 showed symptoms of tiredness, and bloody urine soar in the mouth. Protection and healing of mucosa epithelial cells were prominently displayed by the extract at 200 mg/kg in contrast to the 100 mg/kg, depicting a better ulcer healing capacity, which compared favorably well with the reference drug used.

This finding agrees with the submission of Mohamed *et al* [25] where the gastroprotective potentials of the extract against aspirin-ulcerated rats and curative potential in alcohol-induced rats were associated with their polyphenolic compound and other various bioactive compounds. Since Omeprazole is a proton pump inhibitor, then the effects produced by the three extracts might have perhaps mimicked its mechanisms of action by modulating cells in the mucosal lining of the stomach against excess acid secretion.

Limitations of this study

One limitation of this study is that the specific mechanism of antimicrobial and anti-ulcer action of the bioactive molecules was not ascertained, thus warranting its investigation in a future study. Secondly, this study did not determine the influence of genetic factors relating to the genes that were upregulated or downregulated following the oral administration of secondary metabolites. Looking at these in future studies will help in the understanding of the overall benefit of the *Streptomyces*-derived bioactive molecules.

CONCLUSION

Streptomyces spp produces a wide range of secondary metabolites that are not toxic to the epithelial tissue and inhibit the growth of pathogenic organisms. In addition, the secondary metabolites possess anti-ulcer activities, an observation further highlighting its potential benefit as an important source of curative, non-harmful drugs against peptic ulcers.

DECLARATIONS

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Ethical approval

Ethical approval (authorization no. MOH/KGS/1376/1/89) for the study was obtained from the Kogi State of Nigeria Ministry of Health Ethics Committee in line with the Code of Conduct for Biomedical Research involving animal subjects.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Conceptualization: Martin-Luther O Okolo, David A Zakari and Kizito E Bello; Field Work, laboratory experiment and data analysis: Kizito E Bello and David A Zakari; Writing Original Draft: David A Zakari and Cornelius A Omatola; Supervision: Martin-Luther O Okolo. Review and editing and approval of the final draft: Cornelius A Omatola, Martin-Luther O Okolo, Sunday A Adegoke, David A Zakari and Kizito E Bello,

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