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Extract and Fractions from Soil Bacteria (*Streptomyces canus* ATCC 12647) Possess Antimicrobial and Anti-Oxidative Potential *in vitro*

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ABSTRACT

Streptomyces species are the most prolific producers of antibiotics within the group actinobacteria. The *in-vitro* antimicrobial and antioxidant activities of the methanol (MeOH) extract and vacuum liquid chromatography (VLC) fractions of a soil bacteria *Streptomyces canus* ATCC 12647 were evaluated. Agar well diffusion method was used for the antimicrobial assay, while phosphomolybdate and DPPH radical scavenging methods were used for the antioxidant assay. The antimicrobial assay showed remarkable activities against *Bacillus subtilis, Staphylococcus aureus,* and *Candida albicans*. Also, the extract and fractions showed good *in-vitro* antioxidant activities in both models. Our results showed that extract and VLC fractions from bacterial isolate had good antimicrobial and antioxidant activities.

Keywords: *Streptomyces canus,* Antimicrobial, Antioxidant, Natural products, Infectious diseases.

1. INTRODUCTION

Human population globally has been devastated by the creasing incidence of infectious diseases, which arose from antimicrobial resistance[1,2]. *Streptomyces* species, characterized by the high level of guanine-cytosine content with the ability to produce bioactive secondary metabolibelongongs to the order *actinomycetale* within the class *actinobacteria* and are among the most important species with diverse gene clusters for the biosynthesis of polyketide, peptides and non-ribosomal peptides[3]. *S. canus* has been identified to produce cyclic depsipeptide telomycin, an antibiotic with noteworthy antibacterial activity[4]. This natural peptide antibiotic exhibits potent *in-vitro* inhibitory activity against gram-positive pathogenic bacteria, including penicillin resistant *Staphylococcus aureus* and vancomycin intermediate *Staphylococcus aureus* (VISA), which are causative agents for hard to treat nosocomial infections[4].

Other isolated bioactive metabolites from *Streptomyces canus* include resistomycin, and tetracenomycin. Resistomycin possesses significant *in vivo* antifungal activity against rice blast[5]. It also, exhibit strong antifungal activity against *Valsa mali* and *Magnaporthe grisea* with IC_{50} of 1.1 μg/mL and 3.8 μg/mL respectively[5]. Column chromatographic separation of the fermented broth of *S. canus* strain FIM0916 led to the isolation of two lipopeptide amphomycin and aspartocin with aspartocin D and E possessing gram positive antibacterial activities[6].

Infectious diseases impose a great deal of oxidative stress to the patients, and there is established link that mounting oxidative stress modifies the diseases pathogenesis[7]. Oxidative stress causes some harmful effects in the body like lipid peroxidation and oxidative damage to DNA[7]. It also plays roles in the development of atherosclerosis, diabetes, inflammation, neurodegenerative diseases like Alzheimer, Parkinson's, and

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some other physiological diseases like aging[8].

Reactive oxygen species cause cancer[9, 10]. The implication of this link between infectious diseases and oxidative stress diseases is that antioxidant therapy is needed for the treatment of infectious diseases[11]. In view of this, this study proposes to screen the extract and VLC fractions of the soil bacteria; *S. canus* strain ATCC 12647 for their antimicrobial and antioxidant activities. This will provide researchers with good background for full characterization and isolation of antioxidant metabolites from *S. canus* ATCC 12647.

2. Materials and Methods

Materials

Starch soluble (Acros Organics, New Jersey, USA), Sodium nitrite (Alfa Aesar, England), XAD 7HP and XAD 16N (20 – 60 mesh) (Sigma Aldrich, USA), Agar (Formedium LTD, England), Methanol and Acetone (JHD, China), Silica gel, Vitamin C, Instant Ocean (trace element) (Aquarium Systems, Sarrebouurg, France), DPPH (Sigma Aldrich, Germany). Water purified by a Milli-Q purification machine (Millipore Corporation, Bedford, MA, USA) was used for this study.

Instrumentation

Electronic weighing balance (Mettler, Germany), Rotary evaporator (Büchi Rotavapor R-200), Heating mantle (Philip Harris, UK), Water bath (Philip Harris, UK), Vacuum pump, Avanti JXN-26 Centrifuge (Beckman Coulter), Innova 4300 Shaker Incubator with a 2.5 cm orbit diameter (New Brunswick Scientific), Genevac® EZ-2 Plus (Autur Mckay), Laminar flow cabinet (BH-EN 2004), UV/Visible Spectrophotometer (Shimadzu, Japan) and -80ºC Freezer (Forma Scientific).

Test Microorganisms

The test microorganisms were type cultures stocked in the culture maintenance unit of the Department of Microbiology, University of Nigeria, Nsukka. Gram-positive bacteria; *Staphylococcus aureus* (ATCC 9027), *Bacillus subtilis* (ATCC 35021). Gram-negative bacteria; *Salmonella typhi* (MTCC-531) *Escherichia coli* (ATCC 6538P), and fungi such as *Candida albicans* (MTCC-183) and *Aspergillus niger* (MTCC 961) were used. Microorganisms were maintained by weekly sub-culturing and incubation at 37ºC for bacteria and 25ºC for fungi. Twenty-four-hour culture of each text organisms was used for the assay.

Sample Collection

S. canus is a terrestrial actinomycete isolated from soil in the USA. The *S. canus* strain ATCC 12647 was provided by Prof. RJM Goss, School of Chemistry, University of St Andrews, United Kingdom. Mycelial stocks were preserved in 25% glycerol and stored in a refrigerator maintained at - 80ºC until needed for use.

Culturing Procedures

Starter culture

Starter culture of *S. canus* ATCC 12647 strain was grown on 100 mL (2 *x* 50 mL) volumes of M5 media (0.20 g of soluble starch, 0.01 g of $NaNO_2$, 0.05 g of K_2HPO_4, 0.05 g of $MgSO_4$, 0.15 g of agar, 1000 μL trace element, 100 mL of MilliQ water) for 4 days at 28ºC and 180 rpm. The M5 medium was autoclaved for 20 min at 121°C load temperature before use.

Main culture

The main culture of *Streptomyces canus* strain was grown on 10 L (20 *x* 500 mL) volumes of M5 media (20 g of soluble starch, 1 g of $NaNO_2$, 5 g of K_2HPO_4, 5 g of $MgSO_4$, 1000 μL trace element, 1 L of MilliQ water) with agitation for 7 days at 180 rpm in an Innova 4300 shaker incubator.

Extraction and Purification Procedure

After fermentation of the main culture, broth was centrifuged at 8000 rpm for 1 h at a temperature of 4ºC and the supernatant mixed with XAD-7HP and XAD-16N (1:1, 10% *w/v*), agitated continuously for 7 h and filtered using sintered glass funnel. The resin was then washed with 10.0 L MilliQ water, and extracted with methanol (5.0 L). The solvent was removed at a reduced temperature and pressure using a rotary evaporator to yield the extract[12]. The dry extract was purified using vacuum liquid chromatography (VLC). Briefly, the dried extract (10.8 g) was triturated with silica gel (10.0 g) in a mortar and loaded onto a sintered glass Buckner funnel (6 cm *x* 30 cm, ID) attached to a vacuum line and

packed with graded silica gel 60 (0.04-0.063 mm, 230-400 mesh) as adsorbent, then eluted with methanol in acetone gradient (25, 50, 75 and 100%, 1 L each) to yield the VLC fractions (F1-F4). These sub-fractions were subsequently concentrated to remove the solvents and used for the antimicrobial and antioxidant studies[13].

Antimicrobial Screening of Extract and VLC Fractions

To assay the antimicrobial activity of the extract and sub-fractions, well diffusion method was used[14,15]. Bacteria and fungi were seeded uniformly in nutrient agar and incubated for 24 h at 37ºC and 27ºC respectively, and 10 mL of nutrient agar was inoculated with the single colony formed. The culture was incubated in a Laminar flow cabinet for 24 h. After the incubation a 10% of the inoculum was used to inoculate a 0.5% of Muller-Hinton agar which has been cooled down to 40ºC and then transferred into an agar plate with a cork-borer of 6 mm in diameter. The extract and fractions were diluted two-fold (10, 5, 2.5, 1.25 mg/mL) using DMSO and10 µL volumes were loaded onto each disc with ciprofloxacin and fluconazole as positive controls respectively and DMSO as negative control. Agar plates were incubated at 37ºC for bacteria and 27ºC for fungi and the inhibition zone diameter determined after 24 h and 48 h of incubation respectively. A linear plot of square of inhibition zone diameter (IZD^2) against log concentration to base 10 was made and the minimum inhibitory concentration (MIC) values of the samples determined as the zero intercept of the linear regression[16].

Antioxidant Assay

Phosphomolybdate and DPPH radical scavenging activity methods were used for *in-vitro* antioxidant assay of the extract and VLC factions.

DPPH Radical Scavenging Method

Free radical scavenging activity of extract and VLC fractions were determined using 1, 1-diphenyl-2-picryhydrazyl (DPPH) method[17]. Briefly, 0.1 mL solution of DPPH (4.5 mg/100 mL) in methanol was added to 3 mL of different concentrations (10, 20, 30, and 40 mg/mL) of the samples dissolved in methanol in ependorf vials. The mixture was agitated vigorously and incubated at room temperature for 30 min. Then, the absorbance of mixtures was measured at 517 nm by using spectrophotometer (UV-VIS Shimadzu). Control solution was prepared by mixing 3.5 mL methanol and 0.3 mL DPPH radical solution. Ascorbic acid was used as reference antioxidant compound and the experiment was done in triplicate. The percentage inhibition of the DPPH scavenging activity was calculated using the formula below:

$$Percentage\ inhibition = (1 - \frac{A_1}{A_0})\ x\ 100$$

Where: A_0 is the absorbance of the control and A_1 is the absorbance of the test samples.

Total Antioxidant Capacity Assay (TAC) by Phosphomolybdate Method

The total antioxidant capacity assay of extract and VLC fractions was determined by the phosphomolybdate method[18]. Briefly, 0.1 mL aliquot of the various concentrations (10, 20, 30 40, and 50 mg/mL) of the samples were mixed with 1.0 mL of reagent solution (600 mM of H_2SO_4, 28 mM of Na_3PO_4, and 4 mM ammonium molybdate, 1:1:1) in test tubes and incubated in a water bath at 95ºC for 1.5 h, then cooled to room temperature and the absorbance of mixture was determined at 765 nm against a blank containing 1 mL of the reagent solution. Ascorbic acid was used as positive control. The assay was carried out in triplicate and the total antioxidant capacity was calculated using the formula below:

$$Percentage\ TAC = (1 - \frac{A}{A_0})\ x\ 100$$

Where A_0 is the absorbance of the blank; A is the absorbance of the test samples.

Statistical analysis

The results were expressed as mean ± standard deviation (n = 3) and analyzed using descriptive statistics.

3. Results

Antimicrobial screening

All tested samples exhibited good antimicrobial activity against tested micro-organisms. F4 showed prominent activity against four of the tested organisms while F1 and F2 exhibited good inhibitory activity against *C. albicans* and *S. aureus* respectively (Table 1).

Table 1. Minimum Inhibitory Concentrations of the Extract and VLC Fractions

	Minimum Inhibitory Concentrations (MICs) (µg/mL)					
	A. niger	***C. albicans***	***E. coli***	***S. typhi***	***B. subtilis***	***S. aureus***
Extract	985±0.020	740±0.031	1687±0.031	1049±0.011	1388±0.031	1055±0.022
F1	1732±0.011	197±0.014	1442±0.025	1353±0.013	1252±0.012	1426±0.041
F2	1222±0.033	1278±0.031	1297±0.015	1443±0.012	796±0.022	801±0.028
F3	714±0.022	1104±0.023	816±0.023	453±0.023	855±0.011	417±0.022
F4	435±0.031	841±0.042	336±0.032	168±0.014	633±0.015	1886±0.032
CPF	ND	ND	1.040±0.033	1.637±0.213	697±0.033	594±0.026
FCZ	0.380±0.034	0.144±0.031	ND	ND	ND	ND

F1 – F 4 = solvent fractions, CPF = ciprofloxacin, FCZ = fluconazole, ND = Not tested

Antioxidant Assay

The methanol extract and VLC factions of *S. canus* ATCC 12647 showed potent antioxidant activity. The percent antioxidant inhibition obtained from both models were dose dependent. However, the radical scavenging potentials of the extract and fractions were remarkably higher than that of the total antioxidant capacity since DPPH assay s more sensitive than Phosphomolybdate Method (Table 2 & 3).

Table 2. Radical Scavenging Activity (%) of the Extract and VLC Fractions

	% Inhibition of Samples at different Concentration (mg/mL)				
	10	**20**	**30**	**40**	**50**
Extract	36.71±0.03	41.18±0.10	48.55±0.06	64.06±0.96	64.93±0.14
F1	22.00±0.03	31.91±0.08	36.05±0.26	47.19±0.01	55.49±0.09
F2	17.70±0.12	26.67±0.09	37.02±0.06	38.36±0.09	40.37±0.40
F3	35.33±0.02	37.73±0.12	42.64±0.07	47.37±0.04	60.66±0.04
F4	18.32±0.12	23.58±0.08	36.59±0.22	38.21±0.13	47.90±0.10
Ascorbic Acid	48.31±0.21	64.51±0.16	67.71±0.20	70.42±0.23	72.80±0.24

F1 – F4 = solvent fractions

Table 3. Total Antioxidant Capacity (%) of the Extract and VLC Fractions

	% Inhibition of Samples at different Concentration (mg/mL)				
	10	**20**	**30**	**40**	**50**
Extract	5.21±0.04	10.52±0.01	15.89±0.05	20.85±0.07	30.05±0.08
F1	2.46±0.02	7.41±0.02	15.39±0.02	25.42±0.05	31.93±0.04
F2	0.26±0.01	1.02±0.02	6.17±0.02	14.89±0.01	19.10±0.03
F3	3.30±0.20	5.40±0.08	14.10±0.11	25.00±0.17	26.01±0.04
F4	6.52±0.01	13.71±0.02	19.13±0.04	25.38±0.02	34.72±0.03
Ascorbic Acid	9.38±0.04	19.67±0.33	25.82±0.02	32.99±0.01	44.14±0.83

F1 – F4 = solvent fractions

4. Discussion

Antimicrobial Activity

The genus *Streptomyces* are renowned producers of potent bioactive natural metabolites such as antifungals, antivirals, antitumor, antihypertensive, antioxidants, immunosuppressant especially antibiotics[19,20] and the inhibitory potency of their metabolites has been reported[21]. Today, approximately 80% of the antibiotics are gotten from *Streptomyces*[22] with over 50% clinically useful[23]. *Streptomyces* through this capability of producing these diverse chemical scaffolds which confers wide ranges of biological activity have contributed significantly to mankind[24,25]. *S. canus* ATCC 12647 is a prolific member of soil actinobacteria which produces ranges of metabolites, prominent of which is telomycin and its analogues. These metabolites exhibit strong bactericidal effect and are effective against lots of multidrug resistant Gram-positive pathogens[4]. The antimicrobial screening of extract and VLC fractions showed that it has good antimicrobial activity against the tested pathogenic organisms with all the fractions exhibiting good antibacterial and antifungal activities. F4 showed prominent activity against the tested organisms with MIC value of 168±0.014 µg/mL against *S. typhi* and 1886±0.032 µg/mL against *S. aureus.* F4 also demonstrated good inhibitory activity against *A. niger, C. albicans, E. coli and B. subtilis* than the other fractions or extract. F1 showed good inhibitory activity against *C. albicans* with MIC value of 197±0.014 µg/mL whereas F4 was most activity against *A. niger* (MIC = 435±0.031 µg/mL). However, fluconazole, elicited better antifungal activity than any of the fractions, with MIC values 0.380±0.034 µg/mL and 0.144±0.031 µg/mL respectively for *A. niger* and *C. albicans*. F3 showed most activity against *S. aureus* with MIC value of 417±0.022 µg/mL. All the fractions elicited good antibacterial activity against the tested bacteria pathogens with the standard drug (ciprofloxacin), having highest activity against *S. aureus* with MIC 0.594±0.026 µg/mL (Table 1).

Antioxidant Assay

The free radical scavenging activity of the extract and fractions was evaluated using the 1, 1-diphenyl-2-picryhydrazyl (DPPH) assay while the total antioxidant capacity (TAC) assay was carried out using the phosphomolybdate model. The results of these *in-vitro* models showed that the extract and VLC factions of *S. canus* ATCC 12647 have remarkable antioxidant activity. The percent antioxidant inhibitions obtained from both models were dose dependent. Our results showed that the extract produced higher antioxidant activity (64.93±0.14%) than the VLC fractions in the radical scavenging assay. This indicated that the bioactive metabolites have synergistic antioxidant potentials. Studies has shown that *S. canus* produces antibiotic like telomycin, vancomycin and resistomycin, no report of synergistic antioxidant activity of these biomolecules have been documented. But synergistic antioxidant activity of plant metabolites isolated from different plants has been reported[26]. These plant metabolites isolated from different plants belong to the polyphenolics and their antioxidant activities could be attributed to the radical scavenging potentials of polyphenolic compounds.

Compared to the ascorbic acid with percent inhibition of 72.80±0.24, all the fractions elicited remarkable antioxidant activity with F3 having the highest free radical scavenging activity (inhibition percentage = 60.66±0.04) (Table 2). Similar dose dependent antioxidant effects were also observed in the phosphomolybdate model. In this model, F1 produce higher percentage inhibition. Comparing the two models, the 1, 1-diphenyl-2-picryhydrazyl (DPPH) seems to be more sensitive than the phosphomolybdate method. This is evident in the percentage antioxidant inhibition elicited from both models. DPPH assay s more sensitive than the phosphomolybdate method since its radical scavenging activity involves donation of hydrogen atom or transfer of an electron to the nitrogen atom to scavenge the radical unlike the phosphomolybdate method[27-29].

5. Conclusion

Our results showed that extract and VLC fractions of *Streptomyces canus* ATCC 12467 demonstrated good *in-vitro* antimicrobial activity, and with remarkable antioxidant potency. Detailed and elaborate activity guided isolation, and characterization of bioactive metabolites from the most active fractions is on-going.

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Conflict of interests

We declare that there is no conflict of interest.

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يمتلك المستخلص والكسور من بكتيريا التربة (*Streptomyces canus* ATCC 12647) إمكانات مضادات الميكروبات ومضادات الأكسدة في المختبر

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ملخص

أنواع Streptomyces هي أكثر منتجي المضادات الحيوية غزارة ضمن مجموعة البكتيريا الشعاعية. تم تقييم أنشطة مضادات الميكروبات ومضادات الأكسدة في المختبر لمستخلص الميثانول (MeOH) وأجزاء الكروماتوجرافيا السائلة الفراغية (VLC) لبكتيريا التربة Streptomyces canus ATCC 12647. تم استخدام طريقة آجار لنشر البئر لفحص مضادات الميكروبات، بينما تم استخدام طرق الكسح الجذري للفوسفوموليبدات و DPPH لفحص مضادات الأكسدة. أظهر اختبار مضادات الميكروبات أنشطة ملحوظة ضد *Bacillus subtilis* و *Staphylococcus aureus* و *Candida albicans*. كما أظهر المستخلص والكسور أنشطة جيدة لمضادات الأكسدة في المختبر في كلا النموذجين. أظهرت نتائجنا أن مستخلص و VLC من العزلة البكتيرية كان لها نشاط جيد كمضاد للميكروبات ومضادات الأكسدة.

الكلمات الدالة: : *Streptomyces canus*، مضادات الميكروبات، مضادات الأكسدة، المنتجات الطبيعية، الأمراض المعدية.

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