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Diisooctyl Phthalate as A Secondary Metabolite from Actinomycete Inhabit Animal's Dung with Promising Antimicrobial Activity

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Abstract

In the present work, actinomycete isolates with potential antibacterial activity were sought from animal manure. Twenty-two actinomycete isolates were isolated and nominated on the basis of their cultural properties on starch nitrate agar medium. The antibacterial activity of these isolates against a variety of bacterial and fungal species was examined. Actinomycete isolate A3 showed the highest antibacterial activity among those examined. The morphological, physiological, biochemical, and molecular characteristics of isolate A3 indicate that it belongs to the strain *Streptomyces marokkonensis* LMG 23016. The optimal conditions for development and antibacterial activity were submerged cultivation at 30 degrees Celsius and a pH of 7 for seven days. The optimal carbon and nitrogen sources are starch and potassium nitrate, respectively, and 1 percent (w/v) sodium chloride. The antimicrobial metabolites of isolate A3 were extracted and analyzed using thin-layer chromatography (TLC), which revealed a solitary spot. UV, IR, HPLC, and GC/MS analysis confirmed diisooctyl phthalate (1,2-benzene dicarboxylic acid, dioctyl ester) to be the major component. The results of the minimum inhibitory concentration and minimum bactericidal concentration tests on the *S. marokkonensis* strain LMG 23016 A3 extract demonstrated a broad-spectrum action against bacteria, thereby creating new opportunities for the development of potent antibiotic candidates.

Keywords: Animals' dung actinomycetes; Diisooctyl phthalate; Streptomyces marokkonensis

1. Introduction

Recent years have shown a rapid increase in the difficulties associated with antibiotic resistance, which now pose a serious threat to people everywhere and are directly responsible for extremely high rates of morbidity and mortality in people [1]. Therefore, researchers worldwide are continually hunting for new antibiotic compounds to tackle the devastating impacts and dynamic nature of antibiotic resistance [2]. Hence, novel antibiotics and antibacterial agents are vital for modern medicine.

The frontier in finding important bioactive chemicals for pharmacological use is microbial natural products [3]. Particularly, animals' dung is a cheap and readily available bioresource on our planet and is a potential gold mine for microbes. Since ancient times, animal dung has been used in traditional medicine, as a biogas source, and to increase crop productivity [4].

Animals' dung is a very humified organic fertilizer that is nutrient-rich and a source of a variety of aerobic microorganisms, including actinomycetes which grow in reaction to variable levels of pH, oxygen, humidity, and temperature. Actinomycetes and other compost-growing microbes are thought to be capable of producing extracellular hydrolytic enzymes and antibacterial chemicals [5]. Indeed, it is possible to identify aminoglycosides, peptides, ansamycins, β-lactams, tetracyclines, macrolides, lincosamides, epoxides, aminocoumarins proteases, and amylases [6].

Actinomycetes are the most prevalent higher bacteria that produce hyphae that resemble fungi, and they are widely dispersed and extremely common in the soil. The variety of actinomycetes is extraordinarily important for the manufacture of antibiotics such as ansamycins, β-lactams, and tetracyclines [7]. More than 80% of the commercially available naturally occurring antibiotics are produced

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by various actinomycetes. They showed in several studies to have a wide range of biomedical applications, including antibacterial [8], antibiofilm [9], antifungal [10], and anticancer [11] activities.

Streptomyces is the dominant of all actinomycetes [12]. They exist in practically every environment, from the deep sea to the highest mountains [13]. Streptomyces are known for producing a variety of extracellular enzymes as well as various bioactive secondary metabolites such as proteases, lipases, tirandamycins, streptozocin, anisomycin, and other secondary metabolites with various structural and functional characteristics that are used as pesticides, herbicides, antibacterial, antiprotozoal, antifungal, antiviral, antihelminthic, anticholesterol, anticancer, and immunosuppressants [14]. Besides, Streptomyces produces bioactive secondary metabolites that have gained significant attention for their beneficial biological activities, particularly for the effects on human health [3].

Therefore, to develop new antibiotics against drug/multi-drug resistant strains, more actinomycetes from various ecosystems will need to be investigated for antimicrobial activity. Because of this, the present study was undertaken to find potential actinomycetes isolated from animals' dung having the ability to produce antimicrobial compounds.

2. Materials and Methods

2.1. Sample collection

Three separate locations in Egypt's Behera Governorate were chosen to collect aseptically samples of animal excrement. The animal dung samples were collected from different animals including cow, horse, rabbit, goat, buffalo, donkey, and sheep dung in May 2019. The samples were all tagged and delivered to the Microbiology lab, Department of Botany and Microbiology, Faculty of Science, Tanta University for additional dispensation. On the same day, the dung samples were further processed.

2.2. Isolation, purification, and maintenance of actinomycetes

The following media are used for the isolation, starch-nitrate agar medium, yeast extract-malt extract agar medium, and nutrient agar medium. Actinomycete isolates were recovered from dung samples using a serial dilution approach [15]. Three plates were used for each sample, and they were incubated for 7 to 14 days at 30 °C. The growth of actinomycetes on the plates was periodically monitored. The colonies that developed were selected and purified based on color, dryness, roughness, and convexity [16]. The pure colonies were chosen, isolated, sub-cultured, purified, and kept at 4 °C in starch nitrate agar slants for further research. The selected isolates were maintained by being suspended in 50% glycerol and stored at -80 °C [17].

2.3. Screening the actinomycetes isolates for antimicrobial activity

The effectiveness of the actinomycetes isolates for antimicrobial activity was tested using some test microorganisms. Nine test bacterial strains (*E. coli* ATTC8739, *Salmonella typhi* ATTC14028, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATTC35639, *Klebsiella pneumonia* ATCC53637, *Proteus* sp ATTC35659, *Staphylococcus epidermidis*, *Bacillus cereus*, and *B. subtilis*) and five test fungal strains (*Fusarium equiseti*, *Fusarium sublutinase*, *Fusarium proliferatum*, *Aspergillus niger*, and *Candida albicans* ATTC90028) were employed to test the isolated actinomycetes for antimicrobial activity.

Preliminary testing of antimicrobial activity of pure actinomycete isolates using an agar plug assay (Cork borer method) [18]. Production of antimicrobial compounds was performed by submerged fermentation according to Egorov [19]. Briefly, under sterile conditions, the most powerful antimicrobial-generating actinomycetes were grown in 50 mL of starch–nitrate broth in a 250 mL capacity conical flask and then incubated at 150 rpm at 30 °C for 7 days. Afterward, to separate cell debris, the media was centrifuged at 10,000 rpm, and the filtrate obtained was used for secondary screening. Based on the results of the agar plug test, the isolates that exhibit antimicrobial activity were subjected to secondary screening using starch nitrate broth using the Agar well method **[**16]. The most effective isolate was selected for further experiments.

2.4. Antibiotic sensitivity profile of tested bacteria

The tested bacterial strains were kindly supplied by Assoc. Prof. Dr. Lamiaa Al-Madboly at the Microbiology Department, Faculty of Pharmacy, Tanta University. According to Clinical and Laboratory Standard Institute (CLSI) guidelines, an antibiotic susceptibility profile of the tested bacteria was performed to confirm the resistant percentage [20]. Antibiotic susceptibility test for the tested bacteria was carried out according to [21, 22]. Transferring 500-1000µL culture broth onto Mueller Hinton agar plates, followed by surface streaking the whole agar surface with a sterile wire loop, resulted in seeded Mueller Hinton agar plates (MHA). The antibiotic discs were aseptically deposited onto the agar surfaces after the seeded

agar plates had been left for around 15 minutes. After that, the plates were incubated at 37 °C for 18-24 h. Inhibition zones were measured and documented in mm.

2.5. Identification of the most active actinomycetes isolates

For complete and useful identification of the selected actinomycete isolate, numerous morphological, chemical, and physical characteristics were investigated. The conditions outlined in the identification keys followed Bergey's manual of systematic bacteriology [23] and Bergey's manual of determinative bacteriology was followed for identification [24]. Morphological characteristics of the actinomycete isolates were studied using inorganic-salt starch agar medium according to the ISP methods [25] as well as the coverslip culture technique [26]. Cell wall analysis was carried out using the methods designated by Becker *et al* [27] and Lechevalier and Lechevalier [28]. Microscopic studies were carried out at the Faculty of Science at Alexandrea University using a cover slip light microscope (Optika, Italy) and a scanning electron microscope (JEOL Technics JSM-IT200, Japan) and molecular identification was carried out in Sigma company, Cairo-Egypt [23, 29].

In the Sigma research lab in Cairo, Egypt, the selected *Streptomyces* sp was molecularly identified. A modified method of preparing genomic DNA was used [30]. My Taq red mix, DNA template, (20 Pico mol) forward primers, (20 Pico mol) reverse primers, and nuclease-free water were all in the reaction mixture, with quantities of 25, 8, 1, 1, and 15 µL, respectively. Initial denaturation at 94 for 6 minutes was followed by denaturation, annealing, and extension at 94, 56, and 72 for 45 seconds, 45 seconds, and 1 minute, respectively. The previous phase took 35 cycles to complete, while the final extension took 5 minutes at 72 degrees. A polymerase chain reaction was used to amplify the 16S rDNA. using universal primers (F: 5' TGAGCCTTGTAAGCGTCCAC 3'; R: 5' TTCATGCCGTGCTTCTCCAG 3') designed to amplify 1500 base pair fragments 16S rDNA region in relation to the *E. coli* genomic DNA sequence by PCR reaction. After the PCR reaction was completed, a fraction of the PCR was evaluated using agarose gel electrophoresis, and the remaining mixture was purified using the EZ-10 spin column DNA gel extraction kit (Bio Basic Inc.) and sequenced using Sigma. After putting the sequences together, they were compared.

2.6. Optimization of cultural conditions on growth and antimicrobial activities of the selected actinomycete isolate

Several factors were investigated, including the effect of the culture method [33], different incubation periods, different pH values, different incubation temperatures, different nitrogen sources [32], different carbon sources [33], and different NaCl concentrations [34] on the growth and antimicrobial activity of the isolate A3. After each incubation period of all parameters, each culture was centrifuged at 3000 rpm for 20 minutes then the supernatant of each parameter was taken to evaluate the antimicrobial activity of the selected isolate against the most sensitive tested microorganisms by using the good diffusion agar method as mentioned before. The biomass of the selected isolate was transferred to a dry filter paper that had been pre-weighed. This filter paper was then put in an oven overnight at 50 °C to achieve a constant weight. Mycelial dry weight was determined and expressed as g/50 mL for each parameter [35]. Three replicates were used for each parameter.

2.7. Extraction of active antimicrobial compounds

The highly active actinomycete isolate was chosen for a further liquid-liquid extraction method with different solvents based on the results of the primary and secondary screening to detect the antimicrobial metabolites [36]. An equal volume of butanol, diethyl ether, petroleum ether, ethyl acetate, hexane, chloroform, acetone, methanol, and ethanol were added to the fermented culture filtrate separately (all solvents were purchased from Sigma Company in Cairo, Egypt) and shaken vigorously for 30 minutes. The organic phase or the precipitate of the culture was obtained, and microbial activity was tested using the agar well diffusion method against tested microorganisms. The optimum solvent for subsequent extraction was chosen based on the maximum inhibition, and the organic phase was evaporated using a rotary evaporator, (SENECO Technology Co., Ltd., Taiwan). The completely dried crude extract was collected and used for further studies.

2.8. Purification and characterization of antimicrobial active compounds in the crude extract

2.8.1 Thin layer chromatography analysis

Thin layer chromatography (TLC) using a silica gel plate was applied to screen and investigate the purity degree of the crude ethyl acetate extract (most active fraction) of the active actinomycete strain [37]. By a capillary tube, the sample was spotted in three different TLC plates. Then, the

plates were investigated in three distinct solvent systems, *i.e.*, water: methanol (4:6, *v/v*), chloroform: methanol (4:6, *v/v*), and chloroform: methanol (9:1, *v/v*) applying the ascending development. Afterward, the plates were visualized after drying by UV lamp Model Spectro line (highest ultraviolet intensity, U.S.A) at 254 nm and 366 nm.

2.8.2 HPLC analysis

Waters Alliance 2695 with Waters PDA detector 2998 (Waters, Milford, MA, USA) was used for HPLC analysis, as described by Ludwig, *et al* [38] with few modifications. In brief, the ethyl acetate residue was re-suspended in HPLC-grade methanol and filtered through a 0.45 µm PTFE disc filter (VWR International, Germany). Following that, the filtrate was chromatographed with a Discovery HS C_{18} (5 µm, 250 mm x 4.6 mm) column combined with a guard column (Phenomenex®) at a constant temperature of 16 °C. At a flow rate of 1 mL/minute, an isocratic elution with methanol:0.1% formic acid (92:8 v/v) was carried out. The injection volume was set at 20 µL and a PDA detector adjusted at 205 nm was used.

2.8.3 UV analysis

The UV analysis of the investigated ethyl acetate residue was recorded by using a quartz cuvette containing the extract in methanol. The UV/Vis spectrum was achieved using pg. instruments, T80 spectrophotometer, United Kingdom in the range of 250 to 500 nm [39].

2.8.4 FTIR analysis

The functional groups present in the partially purified ethyl acetate residue were characterized in the range of 600-4000 cm^{-1} in KBr disc using Fourier-transform infrared spectrometer (FTIR), Model (Tensor 27 Bruker) [40].

2.8.4 GC-MS analysis

The selected isolate's extract was tested using the GC-MS technique, where a gas chromatograph coupled with a mass spectrometer (GC-MS) (Perkin Elmer model, Clarus 580/560S) was used. The following chromatographic conditions were used, where sample input temperature 280 °C; carrier gas, helium; flow rate 1 mL/min; initial temperature 60 °C maintained for 7 minutes, then programmed to 170 °C for 5 minutes by 10 °C/minute, then programmed to 280 °C for 10 minutes by 10 °C/minute; capillary column, HP-5MS, diameter 0.25 mm, length 30 m. The major compounds were tentatively identified in the mass spectrum and their retention times were compared to those of standards [1].

2.9. Determination of the minimum inhibitory concentration (MIC)

MIC was assayed for the purified active ethyl acetate extract of the selected actinomycete isolate A3 using the microtiter plate technique [41]. A serial dilution (100, 50, 25, 12.5, 6.5, 3, 1.5, and 0.75 mg/mL) of ethyl acetate extract was tested against tested bacteria: ***S. aureus*** ATCC 29213, ***K. pneumonia*** ATCC53637, *S. epidermidis*, *B. cereus* and *B. subtilis*. From each dilute 0.1 mL was added to 5 mL of Nutrient broth containing 0.05 % phenol red and added with 10 % glucose (NBPG medium) [42]. One hundred microliters of each concentration were added in a well (96-wells microplate) that already contained 95 µL of NBPG and 5 µL of the test bacterial suspension containing 10^6 CFU/mL. The negative control well contained the same mixture without adding the active compound of ethyl acetate extract. Covered plates were incubated for 24 h at 37 °C. The experiment was carried out twice more. The change in color in the wells was used to determine microbial growth (yellow when there is growth and red when there is no growth). The MIC was considered the lowest concentration of extract that caused no color change [41].

2.10. Determination of minimum bactericidal concentration (MBC)

MBC determined by microtiter broth dilution method: After a 24-hour incubation period at 37 °C, the MBC was determined as the lowest extract concentration that killed 99.9% of the bacterial inoculum. The approach of Ozturk and Ercisli [43] was used to determine MBC. MBC was carried out on a purified active ethyl acetate extract of a selected isolate. Ten microliters were taken from the MIC experiment's (MIC value) well and two wells above it and spread on MHA plates. The colonies were counted after 18–24 h of incubation at 37 °C. The MBC value was defined as the concentration of a sample that yields less than 10 colonies. Three times each experiment was run.

3. Results and Discussion

3.1. Isolation and screening of antimicrobial-producing actinomycetes

Twenty-two different actinomycete isolates were isolated from animals' dung samples. Each isolate was tested against the test microorganisms for its antimicrobial activity. Twelve isolates showed antimicrobial activity against one or more test

organisms. Secondary screening is performed on actinomycete isolates which show antimicrobial activity in primary screening against the tested bacterial and fungal species. Isolate named A3 had the greatest antimicrobial activity as shown in **Table 1**. This made it desirable to identify and study it further.

The isolate A3 established a wide range of antibacterial activity against Gram-negative and Gram-positive bacteria. Compared to Gram-negative bacteria, it demonstrated greater antibacterial efficacy against Gram-positive bacteria. These results were consistent with the previous studies by Omran and Kadhem, [44] and Al-Ghazali and Omran, [45] which found that Gram-negative bacteria were less vulnerable than Gram-positive bacteria to actinomycete-produced antimicrobial compounds. The existence of an outer membrane with hydrophilic lipopolysaccharide chains that acts as an additional barrier to the entry of antibiotic extract into the cells may be the cause of the weak antibacterial action of actinomycetes secondary metabolites against Gram-negative bacteria [44, 46].

3.2. Antibiotic sensitivity profile of tested bacteria

The antibiotic sensitivity profile of investigated bacteria was performed to appoint their resistance percentage to different antibiotics. Twenty-four antibiotics representing different classes of antibiotics were used in the development of the antibiotic sensitivity profile in the form of paper disks (Hi Media company). The results showed that *Salmonella typhi* ATTC14028 was resistant to (70%) of tested antibiotics, *Proteus* sp ATTC35659 (30%), *S. aureus* (47%), *B. cereus* (42%), *S. epedermadides* (31%). However, *B. subtilis* showed the highest susceptibility rate (21%) to tested antibiotics as shown in **Table 2**.

3.3. Identification of the most active actinomycete isolates

According to ISP methods [25, 47], the selected isolate was characterized and identified. According to the results, isolate A3 was discovered to grow successfully on practically all of the tested media. On ISP (2, 3, 5) the isolate displayed good growth. ISP 4 is experiencing very good growth, ISP 7 is experiencing moderate growth, while ISP 1 and 6 are experiencing weak growth. On glycerol-asparagine agar (ISP5) the color of the aerial spore mass is whitish grey while on inorganic salts-starch agar (ISP4), oatmeal agar (ISP3), and yeast extract-malt extract agar (ISP2) is gray. Diffusible pigments are not produced on ISP medium 4 or 5, and melanin pigments are not formed on tryptone-yeast extract agar (ISP 1), peptone-yeast extract-iron agar (ISP 6), or tyrosine agar (ISP 7). The isolate was next examined using a light microscope (Optika, Italy) and a scanning electron microscope (JEOL Technics JSM-IT200, Japan) utilizing the cover slip method to examine the aerial hyphae configurations, spore chain ornamentation, and spore surface. The electron micrographs and visual observations of the isolate showed the morphological characteristics of the organism, as shown in **Figure 1a**.

The data recorded showed that isolate A3 was a Gram-positive, non-motile actinomycete with lengthy spore chains of the spiral type and more than 20 smooth-surfaced, non-flagellated, cylindrical spores. It also develops a branched substrate mycelium. The aerial mycelium is greyish white. The selected isolate was subjected to a variety of physiological and biochemical tests. Data recorded in **Table 3** showed that isolate A3 contains in its cell wall LL-diaminopimelic acid (LL-DAP), so it was characterized among the actinomycetes [48].

Table 1: Secondary screening for antimicrobial activities of the actinomycetes isolates against the tested bacterial and fungal species (Inhibition zone diameter mm).

Tested Organisms / Isolates	Gram positive bacteria				Gram negative bacteria			Fungal species			Yeast species
	B. cereus	*B. subtilis*	*S. aureus* ATCC 29213	*S. epidermidis*	*K. pneumonia* ATCC 53637	*S. typhi* ATTC14028	*Proteus sp* ATTC35659	*A. niger*	*F. equiseiti*	*F. proliferatum*	*C. albicans* ATTC 90028
A3	24±0.25	21±0.2	22±0.25	19±0.4	0±0	20±0.25	20±0.2	0 ±0	0±0	0±0	0±0
A13	16±0.25	0±0	0±0	0±0	0±0	0±0	0±0	30±0.3	22±0.1	25±0.2	20±0.3
A16	18±0.25	14±0.3	16±0.3	0±0	13±0.4	12±0.25	14±0.2	0±0	0±0	12±0.2	0±0
A5	13±0.3	0±0	0±0	0±0	12±0.25	15±0.25	0±0	18±0.25	0±0	0±0	0±0
A18	0±0	0±0	0±0	0±0	0±0	0±0	0±0	19±0.3	0±0	10±0.6	0±0
A9	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	15±0.2
A23	0±0	0±0	13±0.25	0±0	0±0	0±0	0±0	0±0	0±0	13±0	0±0
A21	17±0.4	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
A10	15±0.4	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
A19	0±0	0±0	0±0	0±0	0±0	0±0	0±0	16±0.3	0±0	11±0.3	0±0
A22	0±0	0±0	12±0.25	0±0	0±0	0±0	0±0	0±0	0±0	13±0.1	0±0
A11	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	20±0.4	0±0

Table 2: Antibiotic sensitivity profile of tested bacteria. (Inhibition zone diameter in mm)

Antibiotics	Symbol	Bacterial strains											
		Gram positive								Gram negative			
		S. aureus ATCC 29213		*B. subtilis*		*B. cereus*		*S. epidermidis*		*S. typhi* ATTC14028		*Proteus sp* ATTC35659	
Ciprofloxacin 5 µg	CIP	40	S	40	S	40	S	50	S	30	I	50	S
Gentamycin10 µg	CN	0	R	36	S	40	S	40	S	30	S	30	S
Norfloxacin10 µg	NOR	0	R	40	S	44	S	65	S	0	R	50	S
Cefotaxim30µg	CTX	30	S	44	S	0	R	0	R	0	R	52	S
Co-Trimoxazole25 µg	COT	42	S	50	S	0	R	60	S	44	S	56	S
Cephoxitin30 µg	CX	0	R	0	R	24	S	45	S	30	I	50	S
Telcoplanin30µg	TEC	20	S	34	S	30	S	50	S	-	-	-	-
Linezolid30 µg	LZD	40	S	54	S	0	R	60	S	-	-	-	-
Cefepime30 µg	CPM	-	-	-	-	-	-	-	-	0	R	50	S
Piperacillin Tazobactam 100/10 µg	PIT	-	-	-	-	-	-	-	-	0	R	46	S
Augmentin30 µg	AMC	0	R	30	S	0	R	0	R	-	-	-	-
Ceftazidime30 µg	CAZ	-	-	-	-	-	-	-	-	20	R	46	S
Imipenem10 µg	IPM	-	-	-	-	-	-	-	-	0	R	0	R
Cefuraxime30 µg	CXM	-	-	-	-	-	-	-	-	0	R	24	S
Doxycycline30 µg	DO	50	S	45	S	46	S	52	S	-	-	-	-
Clindamycin 2µg	DA	36	S	32	S	44	S	42	S	-	-	-	-
Vancomycin30 µg	VA	0	R	32	S	30	S	52	S	-	-	-	-
Amikacin30 µg	AK	30	S	38	S	44	S	43	S	32	S	0	R
Erythromycin15 µg	E	38	S	40	S	32	S	50	S	-	-	-	-
Ampicillin10 µg	AMP	0	R	28	S	0	R	0	R	0	R	0	R
Ampicillin sulbactam20 µg	SAM	0	R	30	S	0	R	0	R	0	R	0	R
Meropenem10 µg	MRP	0	R	0	R	0	R	0	R	0	R	20	S
Rifampicin 5µg	RIF	50	S	0	R	26	S	48	S	-	-	-	-
Cefoperazone sulbactam (75/30 µg)	CFS	0	R	0	R	0	R	0	R	0	R	44	S
Resistant percentage %		47%		21%		42%		31%		70%		30%	

Table 3: Physiological, and biochemical characteristics of A3 isolate

Characteristics	Results	Characteristics	Results
detection of diaminopimelic acid (DAP) isomers	LL-DAP	Methyl red (MR) test	-
		Voges-Proskauer (VP) Test	-
Utilization of Carbon sources (2%w/v): Starch	+++	Hydrolysis of: Lipids	+
Sucrose	+	Starch	+
Mannose	++	Casein	+
Glucose	+++	Gelatin	+
Fructose	++	Production of: Catalase	+
Maltose	++	Urease	-
Xylose	+	H_2S	-
Utilization of Nitrogen sources (0.2%w/v):		Citrate utilization	-
Pot. Nitrate	+++	Nitrate reduction	+
Sod. Nitrate	+++	Cellulose degradation	+
Peotone	++	Tolerance to toxic substances:	
Urea	++	Phenol 0.1% (w/v)	S
Yeast extract	++	Sod.azid 0.01% (w/v)	S
Amm. sulfate	+	Sod.azid0.02% (w/v)	S
		Crystal violet% (w/v)	S
Range of pH for growth: 3.0:3.8	-	Sensitivity to some antibiotics: -	
4.0:10	+	Amikacin and Zyvox	S
More than 10	-	Cefoperazone and Augmentin	R
Range of temperature for growth (°C): 10.0	-	Erythromycin and Cefotaxim	S
20.0:40.0	+	Rifampicin and Telcoplanin	S
More than 40	-	Imipenem and Ampicllin	R
Range of Salinity for growth (%w/v): 1.0: 7.0	+	Norfloxacin and Cefuroxime	R
7.0-10.0	wg	Polymixin and Cefotaxim	S
More than 10	-	Ciprofloxacin and Cephoxitin	R
		Meropenem and Vancomycin	S
		Piperacillin and Ceftazidime	R
		Gentamicin and Doxycycline	S

Key index: (+)= Low growth, (++)= Moderate growth, (+++)= High growth, (+)= positive, (-)= negative, (wg)= weak growth, (R)= Resistant, (S) = Sensitive.

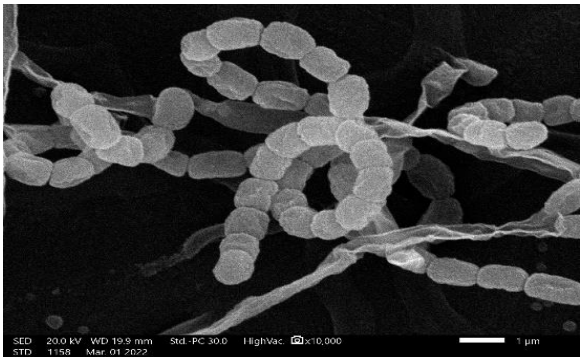

Figure 1a: Scanning electron micrograph (X10000) showing spiralis shaped mycelia and smooth spore surface of isolate A3.

The selected organism suggested belonging to the family *Streptomycetaceae* for the following reasons, their cell wall contains LL-DAP [49]**,** the inability of vegetative mycelia to be fragmented into bacillary or coccoid forms [50], the presence of large spore chains [51], the isolate has excessive branching and aerial mycelia [52].

The isolate A3 can utilize a diversity of sources of carbon and nitrogen. No growth is seen when NaCl concentrations are higher than 10%, and only very weak growth is seen when NaCl concentrations are between 7 and 10%**.** The ideal temperature for growth is 30 °C, with a range of 15 to 40 °C for growth. The pH range for growth is pH 4–10, with pH 7 being the ideal pH for growth. The behavior of the chosen isolate toward various substrates is explained by biochemical features where it could hydrolyze starch and casein, lipids, and gelatin. Nitrate is reduced. H_2S is not produced. Casein and sodium citrate are not utilized, gelatin and cellulose are degraded, methyl red and the Voges-Proskauer test are negative, but the catalase test is positive as shown in **Table 3**.

3.4. Molecular identification of the selected isolate A3

Molecular identification of the most potent antimicrobial producer using 16S rRNA sequencing was carried out. The results indicated the appearance of a single band indicating the purity of isolated RNA. The pure band was partially sequenced and then compared to the public database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) to *Streptomyces* sp. The partial 16S rRNA sequence of the isolates was determined and placed in GenBank under accession number (NR-114960.1) for isolate A3. The selected isolate A3 showed a similarity level of 99% with *Streptomyces marokkonensis* strain LMG 23016 A3 as shown in **Figure 1b**.

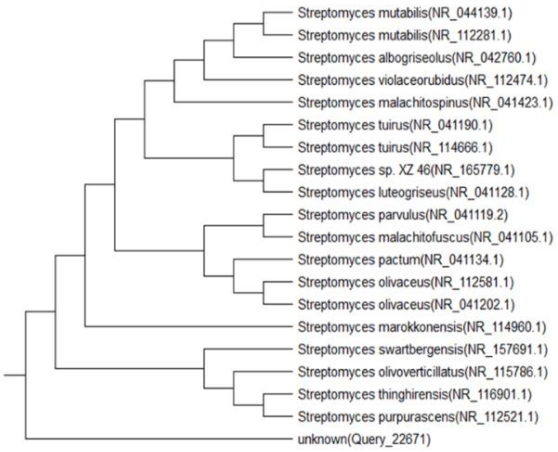

Figure 1b: Phylogenetic tree of isolate *Streptomyces* A3 strain and related *Streptomyces* species based on the 16S rRNA gene sequences

3.5. Optimization of different environmental and nutritional conditions for growth and antimicrobial production from S. marokkonensis LMG 23016 A3

Medium optimization is one of the most critical investigated approaches that was carried out earlier before the 1970s [53]. Optimization of culture conditions was performed to achieve a higher growth rate and antimicrobial production by *Streptomyces marokkonensis* strain LMG 23016 A3 against the most sensitive tested microorganisms.

The results illustrated in **Figure 2a** showed that *S. marokkonensis* LMG 23016 A3's antibacterial activity and growth (dry weight) significantly increased under shaking conditions compared to static conditions. It means that both nutrient availability and aeration achieved by shaking are very significant for growth and metabolic activity of *S. marokkonensis* LMG 23016 A3. Our findings were in line with those of numerous researchers who discovered that shaking culture had higher antimicrobial productivity than static culture. This is because Streptomycetes are obligate aerobic organisms [16].

The incubation period was an effective factor in the growth rate and antimicrobial production of *S. marokkonensis* LMG 23016 A3 which was incubated at different periods in days (1-9). The results in **Figure 2b** showed that there was an increase in the growth in addition to antimicrobial activity with the increase of incubation period from the 1st to the 7th day. However, an additional increase in the incubation period resulted in a decrease in growth and antimicrobial activity. The highest growth rate of the selected isolate and its antibacterial activities on tested bacteria were

obtained on the 7th day of incubation. This result agreed with Shazia *et al* [54]. In contrast, Azish *et al* [55] found that the five-days old culture of *Streptomyces libani* displayed strong antifungal activity against *Aspergillus niger, Aspergillus, fumigatus,* and *Aspergillus flavus*.

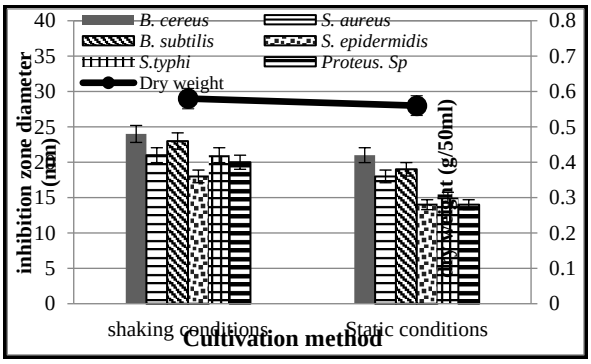

Figure 2a: Effect of different cultivation methods on mycelial dry weight and antibacterial activities of *S. marokkonensis* strain LMG 23016A3 on tested bacterial strains

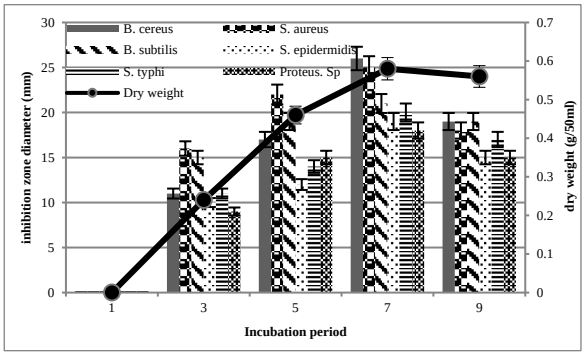

Figure 2b: Effect of different incubation periods on mycelial dry weight and antibacterial activities of *S. marokkonensis* strain LMG 23016A3 on tested bacterial strains

One of the main elements influencing microorganism growth, product production, and metabolic characteristics is the ideal starting pH of the medium. The results represented in **Figure 2c** indicated that the acidic pH 4 is unsuitable for growth or antimicrobial activity for *S. marokkonensis* LMG 23016 A3. On the other hand, the highest growth, and antibacterial activities of *S. marokkonensis* LMG 23016 A3 were recorded at pH value 7 followed by alkaline pH 9 suggesting its presence in the neutrophilic actinomycetes group**.** Low growth and activity were recorded at pH 5 and pH 10. This result is close to that illustrated by Moghannem [16] and in contrast to Fahmy [56] who found that, *Streptomyces sp*. NMF76 grew at a pH ranging from 4-10, the biosynthesis of the antimicrobial agent occurred only under acidic conditions and was maximum at pH 5.

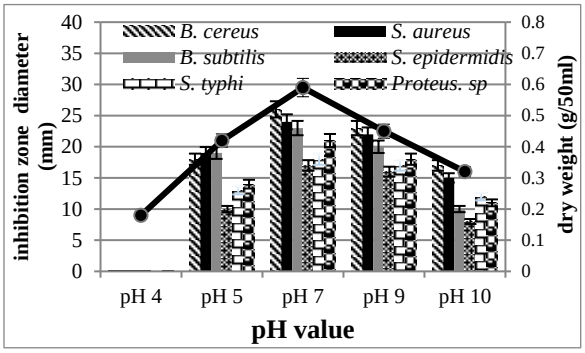

Figure 2c: Effect of different incubation pH values on mycelial dry weight and antibacterial activities of *S. marokkonensis* strain LMG 23016 A3 isolates against the tested bacterial strains

Generally, the majority of *streptomyces* act like neutrophils in culture, and grow best at a pH of between 5.0 and 9.0, with an optimal value that is close to neutrality [57], this might be due to the simplicity with which cells can maintain cytoplasmic pH levels that are almost neutral [58].

Synthesis of antimicrobials was reduced in acidic or highly alkaline environments due to the poor growth of *Streptomyces* species in these pH conditions. The solubility of numerous chemicals required for *Streptomyces* development and productivity is impacted by the pH's extreme alkalinity and acidity [59].

The production of bioactive chemicals and the proliferation of actinomycetes are both significantly influenced by temperature [60]. When the incubation temperature was raised from 20 °C to 30 °C both growth, and antibacterial activity were increased. However, as the temperature rose more (above 30 ºC), growth and antimicrobial activity decreased as shown in **Figure 2d**. *S. marokkonensis* LMG 23016 A3 appeared to be mesophilic in terms of the best temperature for growth. This result agreed with Moghannem, (2018) [16] who showed that *Streptomyces tunisiensis* SA-13 has antibacterial activity between the temperatures of 20 and 35°C, with the maximum activity at 30°C. In contrast, Rakesh *et al* [61] reported that the ideal temperatures for *Streptomyces* sp. mycelial growth and antibiotic synthesis were 45 °C and 40 °C, respectively.

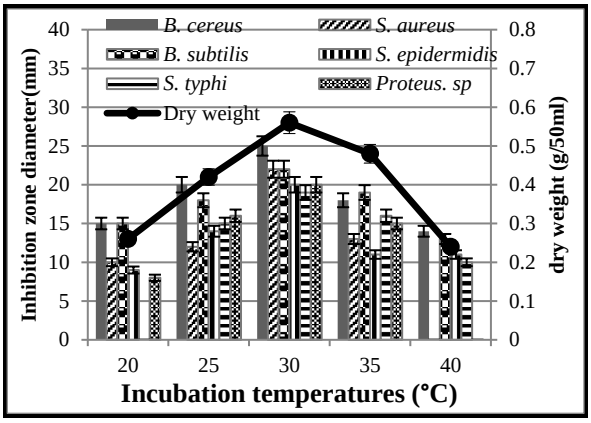

Figure 2d: Effect of different incubation temperatures on mycelial dry weight and antibacterial activities of *S. marokkonensis* strain LMG 23016 A3 against the tested bacterial strains

Among the various tested sources of organic and inorganic nitrogen, the maximum mycelial dry weight, and maximum antimicrobial activity of *S. marokkonensis* LMG 23016 A3 was obtained with potassium nitrate demonstrating that, the type and form of the nitrogen source added to the culture medium may have a significant impact on the amount of antibiotic synthesis. Inorganic nitrogen sources produced *S. marokkonensis* LMG 23016 A3's relatively stronger antibacterial activity when compared to organic nitrogen sources as shown in **Figure 2e**. This result is alike to that obtained by Awadalla *et al* [62] who illustrated that, the maximum mycelial dry weight and antimicrobial activity with potassium nitrate as a nitrogen source. The same result was obtained by Haque *et al* [63] who reported that potassium nitrate was the most suitable nitrogen source for the growth and antimicrobial production of marine *Streptomyces* sp strain HAN-10. This result is not compatible with Singh *et al* [64] found that Soybean meal was the optimum nitrogen source for strain A2D growth and the production of antibacterial agents.

It is well known that the nutritional sources of carbon and nitrogen have a significant impact on the development of antibiotics by actinomycetes [60]. The chosen isolate's capacity to use various carbon sources can significantly impact both its rate of growth and the production of antibacterial compounds.

Figure 2f showed that *S. marokkonensis* LMG 23016 A3 was able to grow in all the carbon sources tested. The best carbon source for the antimicrobial activity of *S. marokkonensis* LMG 23016 A3 was starch against all tested bacteria. *S. marokkonensis* LMG 23016 A3 produced the same biomass in glucose and starch-supplemented medium but not the same antimicrobial activity. A similar result was obtained by Awadalla *et al* [62] who reported that, the maximum mycelial dry weight and significant antimicrobial activity with starch as a carbon source by *S. longisporoflavus*. It has been observed that complex carbon sources like polysaccharides which are slowly assimilated often stimulate secondary metabolite production [65]. In contrast, Fahmy reported that, the maximum antimicrobial activity of *Streptomyces* sp. NMF76 was obtained with glycerol as a carbon source [56].

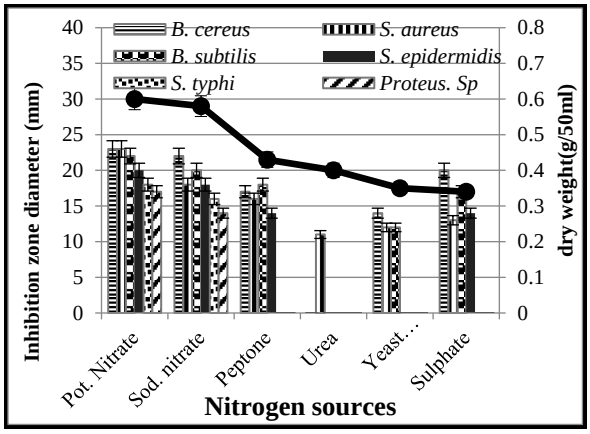

Figure 2e: Effect of different nitrogen sources on mycelial dry weight and antibacterial activities of *S. marokkonensis* strain LMG 23016 A3 against the tested bacterial strains

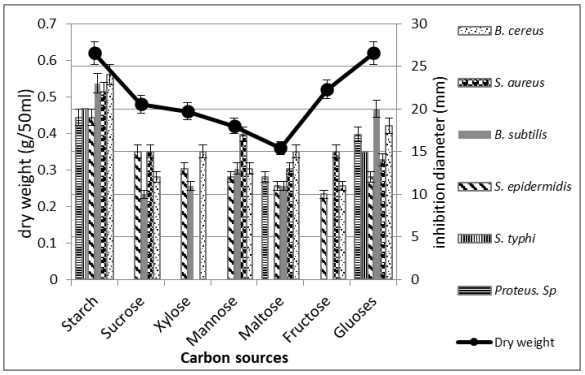

Figure 2f: Effect of different carbon sources on mycelial dry weight and antibacterial activities of *S. marokkonensis* strain LMG 23016 A3 against the tested bacterial strains

The effect of different concentrations of NaCl was studied at (1-15%) giving variable growth rates and antibacterial activities by *S. marokkonensis* LMG 23016 A3. The isolate was estimated for its ability to resist salt stress by growing at NaCl concentrations of 1, 3, 5, 7, and 10%. The highest growth rate and antibacterial activities were obtained at 1 %, and a further increase in salt concentration reduced both the growth rate and the

antimicrobial activities as shown in **Figure 2g**. This result is similar to that obtained by Song *et al* [66] who found that, for *Streptomyces felleus* YJ1, the optimum concentration of NaCl for maximal growth and the formation of antibacterial metabolites was observed to be 1%. However, Fahmy [56] reported that, the maximum antimicrobial activity of *Streptomyces* sp. NMF76 was obtained when cultured in an ISP5 medium containing 3% NaCl.

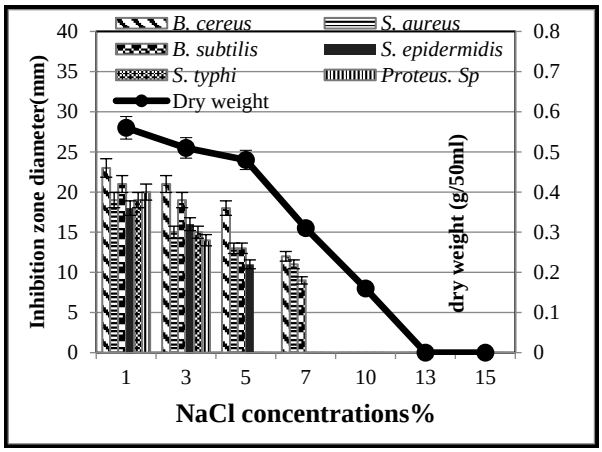

Figure 2g: Effect of different NaCl concentrations on mycelial dry weight and antibacterial activities of *S. marokkonensis* strain LMG 23016 A3 against the tested bacterial strains

Owing to its impact on the osmotic pressure in the medium, the content of salt has an important impact on the production of antibiotics from microorganisms, high salt concentrations tend to denature proteins in addition to influencing osmotic pressure, and obligate halophiles have specialized enzymes that are only active at high salt concentrations [34].

3.6. Extraction of active antimicrobial compounds

It has been stated that organic solvents are always more effective than water-based extraction techniques for extracting chemicals with antimicrobial properties [67]. Notably, ethyl acetate was primarily used as an extraction solvent to separate the crude extracts of bioactive chemicals from actinomycetes in various earlier investigations [40]. In the present study, different solvents were used to extract the active antimicrobial components; ethyl acetate proved to be the most effective antibiotic extraction solvent, this result is alike to that obtained by El-Naggar *et al* [68]; Srivastava and Shanmugaiah, [69] found that, the most effective solvent for producing the most antibiotics was ethyl acetate by *Streptomyces.*

3.7. Purification and characterization of antimicrobial active compounds in the crude extract.

3.7.1. TLC analysis

The separation and purification of antimicrobial crude extract were done using TLC. TLC plates showed one band for ethyl acetate extract with all different solvent systems. TLC analysis of the selected isolate *S. marokkonensis* strain LMG 23016 A3 showed that, the rate of flow (R_f) 0.6, 0.8, and 0.9 with different solvent systems similar results were obtained by Ramani and Kumar, [70] who found that, the antibacterial compounds produced by *Streptomyces* sp Sh7, extracted using butanol and solvent system (Butanol: n-Propanol: Water 40:40:20) and purified by thin layer chromatography had R_f value at 0.6 and also by El-Naggar *et al* [68] who illustrated that, the antimicrobial materials formed by *Streptomyces anulatus* NEAE-94 extracted using ethyl acetate and solvent system ethyl acetate /chloroform, in a ratio of 9:1 had R_f = 0.8.

3.7.2. HPLC

The ethyl acetate extract of the selected isolate was further analyzed by HPLC. The analytical HPLC result was displayed as a single, distinct peak with a retention time of 18.8 minutes. and showed that, the active fractions are extremely pure and impurity-free as shown in **Figure 3a**.

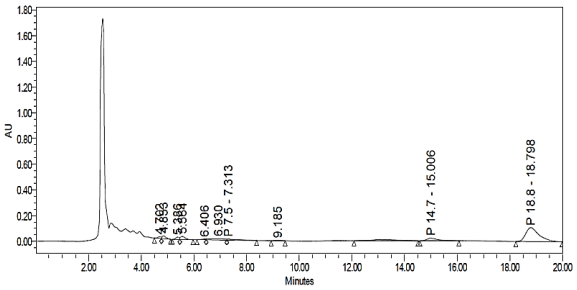

Figure 3a: The HPLC chromatogram of the antimicrobial materials produced by *S. marokkonensis strain* LMG 23016A3

3.7.3. UV spectroscopy

The UV spectrum of the antimicrobial materials of *S. marokkonensis* strain LMG 23016 A3 was found to have a maximum absorption (λ_{max}) at 275 nm as shown in **Figure 3b**. This result is compatible with Rajivgandhi *et al* [39] who reported that, endophytic actinomycetes (EA) *Nocardiopsis* sp. GRG 2 (KT 235641) produced a bioactive substance; 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane (DDIBN) whose UV spectra were visible at 274 nm.

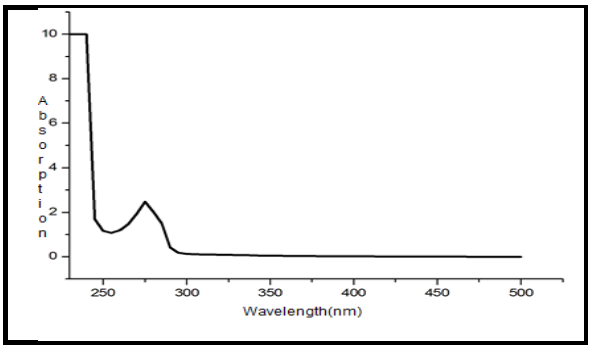

Figure 3b: UV Spectrum of antimicrobial materials produced by *S. marokkonensis strain* LMG 23016A3

3.7.4. IR analysis

The FT-IR approach was confirmed as being essential for elucidating structure, particularly functional groups in chemical compounds [40]. As shown in **Figure 3c** the antimicrobial metabolites generated by *S. marokkonensis* strain LMG 23016 A3 were demonstrated by the FT-IR results to be free of protein or nucleic acid impurities because of the absence of the NH stretching at 3400-3200cm^{-1}. The presence of -C-C alkanes and aromatic groups of CH bending was indicated at the peaks of 685 and 1019 cm^{-1}, and ether bond at 1095 cm^{-1}. Rationally, at 1655 and 2358 cm^{-1}, respectively, the C-H stretching and CH_2 appeared. Alkane groups, C=O of carboxyl stretching, and O-H stretching were all visible at the frequencies of 2924, and 3344 cm^{-1}. The presence of a strong band at 1755cm^{-1} confirmed the presence of the carbonyl group. As a result, our findings showed that the purified fraction contained a carbonyl group. The functional groups listed were most likely to have an antibacterial effect, compared to prior literature [71]. The absence of a toxic cyano group (CN; 2220-2260 cm^{-1}) and acetylenic group (CC; 2100-2260 cm^{-1}) is a sign of the safety of *Streptomyces marokkonensis* extract.

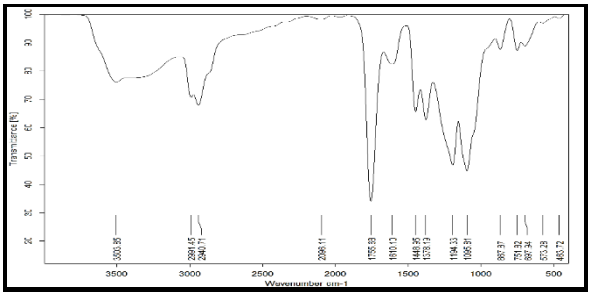

Figure 3c: The infrared spectra (IR) of the antimicrobial materials produced by *S. marokkonensis* strain LMG 23016A3

3.7.5. GC-MS analysis

GC-MS analysis of *S. marokkonensis* strain LMG 23016 A3 ethyl acetate extract fraction showed a single abundant peak, in addition to a few minor compounds at different retention times. The total ion chromatogram of the components of *S. marokkonensis* strain LMG 23016 A3 is illustrated in **Figure 3d** and the list of identified compounds by GC-MS in ethyl acetate extract showed in **Table 3**. The most abundant peak was obtained at a retention time of 32.87 min identified tentatively for diisooctyl phthalate (1,2- benzenedicarboxylic acid, diisooctyl ester) (**Figure 3e**). These results agree with the results obtained by IR analysis for the ethyl acetate residue. It is important to shed light on that, the extraction of diisooctyl phthalate from *S. marokkonensis* strain is detected for the first time in our study. Hence, the broad-spectrum antimicrobial activity against Gram-positive and negative bacteria was highly associated with this metabolite which showed to exhibit antimicrobial activity also in similar previous studies [72]. Furthermore, Dehnad *et al* [73] reported that the antimicrobial effects of extracts of *Streptomyces levis* isolated from northwest soils of Iran could be owing to the presence of the diisooctyl phthalate compound.

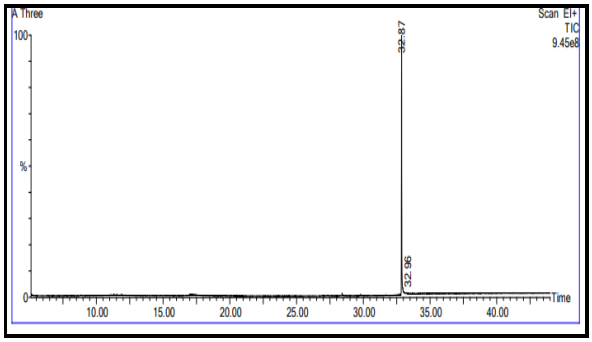

Figure 3d: GC-MS spectrum of the antimicrobial extract produced by *S. marokkonensis strain* LMG 23016A3 showing a single main peak at 32.87 min

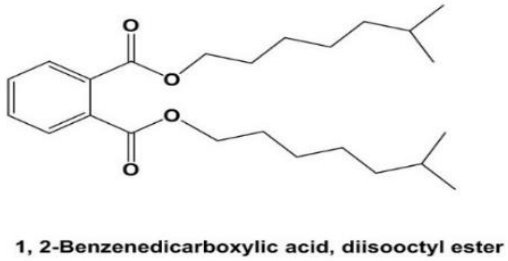

Figure 3e: Chemical structure of 1,2-benzene-dicarboxylic acid, diisooctyl ester (diisooctyl phthalate)

Table 3: List of identified compounds by GC-MS in ethyl acetate extract of *Streptomyces marokkonensis* strain LMG 23016A3

Peak Number	RT	Compound name	Prob	Area%	Library
16	17.023	*1.trans*-2-Decenoic acid	34.5	3.3	MAINLIB
		2.2-Decenoic acid	13.6		MAINLIB
		3.9-Octadecenoic acid (*Z*)-, hexyl ester	3.9		MAINLIB
		4.Dodecyl acrylate	3.3		MAINLIB
		5.Cyclopropaneacetic acid, 2-hexyl-	3.2		MAINLIB
17	17.4	1.1 9,12,15-Octadecatrienoic acid,2[(trimethylsilyl)oxy]1-[[(trimethylsilyl) oxy]methyl]ethyl ester, (*Z*,*Z*,*Z*)-	10.3	0.6	MAINLIB
		2.2 Strychane, 1-acetyl-20*α*-hydroxy-16-methylene-	8.3		MAINLIB
		3. *trans*-2-undecenoic acid	5.0		Replib
		4. *trans*-2-Decenoic acid	4.2		MAINLIB
		5. 2-Decenoic acid	3.3		MAINLIB
24	19.5	1. 4-Amino-1,5-pentandioic acid	6.2	0.59	MAINLIB
		2. *t*-Butyl-{2-[3-(2,2-dimethyl-6-methylenecyclohexyl)- propyl]-[1,3]dithian-2-yl}- dimethyl-silane	5.7		MAINLIB
		3. 2-Thiazolamine, 4-(3,4-dimethoxyphenyl)-5-methyl-	3.5		MAINLIB
		4. *cis*-5,8,11-Eicosatrienoic acid, trimethylsilyl ester	2.8		MAINLIB
		5. Galactoseptanoside, methyl 2,3,4,5-tetra-*O*-methyl-, à-D-	2.6		MAINLIB
26	28.4	1. Phthalic acid, ethyl tetradecyl ester	20.1	0.69	MAINLIB
		2. Phthalic acid, ethyl pentadecyl ester	13.8		MAINLIB
		3. Phthalic acid, ethyl nonyl ester	9.2		MAINLIB
		4. Phthalic acid, dodecyl ethyl ester	8.5		MAINLIB
		5. Phthalic acid, ethyl hexadecyl ester	8.5		MAINLIB
27	32.875	1. 1,2-Benzenedicarboxylic acid, diisooctyl ester	69.3	43.9	replib
		2. 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl)ester	19.6		MAINLIB
		3. Diisooctylphthalate @ P1828 Univ Homburg/Saar	69.3		Pfleger
		4. 1,2-Benzenedicarboxylic acid, diisooctyl ester	69.3		MAINLIB
		5. Di-n-octyl phthalate	4.9		replib

Also, Waheed *et al* [74] found that, extract which contained diisooctyl phthalate exhibited an antimicrobial activity against *Staphylococcus epidermidis, Bacillus subtilis*, *Staphylococcus aureus, Salmonella typhi,* and *Klebsiella pneumonia*.

Diisooctyl phthalate has an antimicrobial and antifouling effect. In addition, this compound has other industrial uses, like as an industrial chemical in polymers to add flexibility to polyvinyl chloride (PVC) resins. They are frequently used for creating wire insulation and as synthetic base stocks for lubricating lubricants. Other applications listed include plasticizers for vinyl, cellulose, acrylate resins, and synthetic rubber. Additionally, diisooctyl phthalate is employed in the production of conveyor belts, tarpaulin, pool liners, flooring, building wire jackets, garden hoses, and automotive hoses and parts. It was also identified in commercial milk products and some children's toys [75].

3.8. Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of purified extract

MIC is defined as the lowest concentration of a chemical that prevents the visible growth of a bacterium. While MBC was recorded as the lowest extract concentration killing 99.9% of the bacterial inoculum after 24 h incubation at 37 °C. In our study, the result revealed that MICs of the bioactive antimicrobial materials of *S. marokkonensis* strain LMG 23016 A3 against *B. cereus*, *S. aureus* ATCC 29213, *B. subtilis*, *S. epidermidis*, *S. Typhi* ATTC14028 and *Proteus* sp ATTC35659 were 1.6, 3.1, 25, 3.1, 25, and 25 mg/mL respectively. While the MBCs of extract of *S. marokkonensis* strain

LMG 23016 A3 against *B. cereus*, *S. aureus* ATCC 29213, *B. subtilis*, *S. epidermidis*, *S. Typhi* ATTC14028 and *Proteus* sp ATTC35659 were 1.6, 6.3, 50, 12.5, 50, and 50 mg/mL respectively as shown in **Table 4**.

Actinomycetes that were isolated from undiscovered areas of the Sundarbans mangrove ecosystem had similar antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis,* and *Salmonella typhi* with MIC values ranging between 0.5 and 5 mg/mL with *Bacillus subtilis* and *Salmonella typhi* and MIC value ranging between 0.05-5 mg/mL with *Staphylococcus aureus* [76].

Table 4: Minimum inhibition concentration (MIC)and minimum bactericidal concentration (MBC) of extract of *S. marokkonensis* strain LMG 23016 A3 against tested bacteria

Tested bacterial species	MIC (mg/mL)	MBC (mg/mL)
B. cereus	1.6	1.6
S. aureus ATCC 29213	3.1	6.3
B. subtilis	25	50
S. epidermidis	3.1	12.5
S. typhi ATTC14028	25	50
Proteus sp ATTC35659	25	50

Kurnianto *et al* [77] reported that broad-spectrum inhibitory activities of the metabolite-crude-extract from the *Streptomyces* isolates exhibited against *Staphylococcus aureus* ATCC 25923, *B. cereus* ATCC 10876, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* InaCC B52 with MIC and MBC ranging from 2.5–10 mg/mL and 5–10 mg/mL, respectively. Moreover, Taechowisan *et al* [78] illustrated that, the crude extract of *Streptomyces* sp. W08f have antibacterial activity against *B. cereus* ATCC 7064, *B. subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, methicillin-resistant *Staphylococcus aureus* Sp6, *Pseudomonas aeruginosa* ATCC 28753, and *S. aureus* ATCC 25923 with MICs and MBCs of 32–128 µg/mL and 256 to >512 µg/mL, respectively.

The different antibacterial activities of the test bacteria were probably caused by variations in the cell walls' composition and structure. Due to the proximity of peptidoglycan polymers to the cell surface of Gram-positive bacteria, antibacterial agents can easily enter the cells. This is distinct from Gram-negative because it contains a lipopolysaccharide-based outer membrane that serves as a barrier to hydrophilic and hydrophobic substances with certain molecular weights [77].

4. Conclusion

Conclusively, there is an urgent need to discover new and potential antibiotics since there is a possibility of the advent of infections that are drug or multi-drug resistant. Then, an important field of research continues to be the identification and isolation of promising actinomycetes with potential antibiotics. The current work is a further investigation of our previous studies on animals' dung for isolation and identification of novel actinomycete for discovery of antimicrobial candidates [79]. The results of the present research demonstrated that actinomycete isolate, *i.e.*, *S. marokkonensis* isolate LMG 23016, from animals' dung samples can produce an antibacterial metabolite that is effective against various pathogens. Chromatographic and spectroscopic-based investigation showed that diisooctyl phthalate was identified to be responsible for such activity. These findings suggest that this fraction may have some therapeutic promise in the treatment of some life-threatening bacterial illnesses. As a result, further efforts must be made to screen animal waste because it has a tremendous potential to yield novel bioactive substances that will help in the development of new medications. Antimicrobial agents can be investigated for their possible uses in the management of human diseases. Finally, the underlined antimicrobial mechanism of action of the bioactive diisooctyl phthalate shall be investigated in further investigations.

5. Conflict of interest

The authors declare no conflict of interest.

6. Acknowledgments

The authors are grateful to **Assoc. Prof. Dr. Lamiaa Al-Madboly** at the Microbiology Department, Faculty of Pharmacy, Tanta University, who kindly supplied us by the tested bacterial strains.

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