ISSN: 2639-7536

Research Article

Petroleum and Chemical Industry International

Production and Characterization of Semi-Quinolone Antibiotic Produced by Streptomyces Griseorubens

Waleed M. Abdulkhair[1],* and Mousa A. Alghuthaymi[2]

[1]*National Organization for Drug Control and Research (NODCAR), Microbiology Department, Giza, Postcode 12553, Egypt.*

[2]*Science and Humanities College, Shaqra, University, Biology Department, Shaqra, Postcode 15517, Saudi Arabia.*

***Corresponding author**
Waleed M. Abdulkhair, National Organization for Drug Control and Research (NODCAR), Microbiology Department, Giza, Postcode 12553, Egypt.

Submitted: 12 May 2022; **Accepted**: 27 May 2022; **Published**: 28 Jul 2022

Citation: *Abdulkhair. WM., Alghuthaymi. MA. (2022). Production and Characterization of Semi-Quinolone Antibiotic Produced by Streptomyces Griseorubens. Petro Chem Indus Intern, 5(3), 137-149.*

Abstract

This work is an attempt to overcome antimicrobial resistance problem which dispersed worldwide in particular developing countries due to misuse of antibiotics. Actinobacteria were isolated and screened against selected resistant Gram-negative bacteria to detect the powerful antibacterial activity. Identification of the most potent actinobacterial isolate has been carried out using classical and genetical methods. Antibacterial compound has been extracted, purified and characterized using accurate and more specific techniques and instruments. Among forty actinobacterial isolates, only twenty-two isolates could inhibit the growth of Gram-negative bacteria. The most potent isolate Eg-7 was identified as S. griseorubens, which has a typical 16S rRNA gene. The antibacterial compound was extracted using ethyl acetate, and separated by High Performance Liquid Chromatography using methanol and water as a mobile phase. Five active peaks were displayed and retained in the range 40 – 45 min, but the last three peaks were retained at 41.90, 43.43, and 44.54 min, respectively. The crude extract was analyzed by liquid chromatography mass spectrum, where the active peak was displayed at 721.325 m/z. The antibacterial compound was purified using flash column chromatography and gel filtration column chromatography. The active fraction was analyzed by Infra-Red spectrum, where a broad absorption at 3338 cm-1 was displayed. Molecular formula of an antibacterial compound was determined by mass spectrum as C35H26N6O4. Nuclear magnetic resonance analysis was carried out for an antibacterial compound. These results suggest that a new antibacterial compound that similar quinolone could be produced by S. griseorubens and exhibited a higher activity against Gram-negative bacteria.

Introduction

The chemical structure of quinolones contains a bicyclic core (Fig. 1) such as nalidixic acid that was discovered in 1960s and used in the treatment of diarrhoea and urinary tract infections caused by *Shigella* spp and Escherichia coli [1]. Nalidixic acid is narrow spectrum antibiotic due to low serum concentration, high minimum inhibitory concentration (MIC) and adverse events [2-4]. Serious bacterial diseases are completely cured with different generations of quinolones due to their highly antibacterial activity (Table 1). Fluoroquinolones are improved derivatives of quinolones due to developed pharmacokinetics which provide a broader spectrum [5]. Fluoroquinolones like ciprofloxacin and ofloxacin are the first choice and highly recommended antibiotics due to highly potency, highly bioavailability, available formulations, highly serum concentrations and mild side effects. Fluoroquinolones inhibit both Gram-negative and Gram-positive bacteria, so they are widely used in the treatment of tuberculosis, anthraxes and other serious bacterial diseases [6]. Rarely, fluoroquinolones affect gastrointestinal tract, nervous system, genome, and eye [7].

Quinolones retards bacterial DNA synthesis via destruction of topoisomerase type II and inhibition of DNA gyrase and topoisomerase IV, which regulate the chromosomal supercoiling [8, 9]. Mutations in the binding sites of DNA gyrase and topoisomerase IV, or an acquisition of a resistant plasmid from other sources are the main reasons for emergence of quinolones resistance by bacteria [10, 12]. Addition of a fluorine atom at R_6 position of quinolone provided a broad-spectrum antibiotic called fluoroquinolone that could inhibit the growth quinolone-resistant bacteria [13]. Ciprofloxacin inhibits the growth of Gram-negative bacteria like *Pseudomonas* spp, while fluoroquinolones which have a piperazine ring at R7 position and substituent of a cyclopropyl group at the R1 could inhibit Gram-positive and Gram-negative bacteria [14].

Many improved pharmacokinetics of fluoroquinolones are present including high absorption, maximum serum concentration (C_{max}), high efficacy at low doses, and safe output away of renal clearance

[15], while the output of quinolones is carrying out by metabolism and renal clearance. Two factors control the efficacy of quinolones; AUC/MIC and C_{max}/MIC, where AUC is the area under the curve. The value of AUC/MIC indicates the efficacy of quinolone, which be high at >125. The value of C_{max}/MIC indicates the antibiotic capacity to overcome a bacterial resistance, and therefore a high C_{max} value over low MIC value is meaning a high antibiotic efficacy, which is usually > 4.0 [16, 17]. For example, fourth generation of quinolones is more effective than other generations due to its highest value of C_{max}/MIC [18, 19]. The improvement of quinolones pharmacokinetics is usually performed by modifications at R_5, R_6, R_7, or R_8 positions. The improved quinolones have longer output half-life, better tissue penetration, increased volume distribution, and better bioavailability. For example, sparfloxacin is an improved quinolone due to addition of an amino group to R_5 position, where the lipophilicity and therefore better tissue penetration are increased. Also, the addition of fluorine atom to R_6 position of quinolone lead to easy penetration to the bacterial cell and increased the volume of distribution [20].

Although quinolones are effective antibiotics particularly fourth generation, some side effects are being associated, such as gastrointestinal disorders and arthralgia. Therefore, they are prescribing to children more than adults. Some side effects are accompanied with earlier quinolones due to narrow spectrum activity and low pharmacokinetics. Prolonged period of quinolone use leads to permanent side effects such as tendon rupture, nerve damage, and fluoroquinolone-associated disability syndrome. Phototoxicity was observed with quinolones combination such as combination of clinafloxacin and sparfloxacin. Specific side effects were observed, such as hematological toxicity with temafloxacin, hepatitis with trovafloxacin, and hypoglycemia with clinafloxacin and gatifloxacin. Immunological side effects were also observed which represented in the disorders of central nervous system and genotoxicity, which is usually observed with some fluoroquinolones when exposed to ultraviolet light, such as lomefloxacin, ciprofloxacin, and moxifloxacin. Although quinolones side effects, they are widely used because their toxicity is reduced due to structural modifications. Nevertheless, there warmings were introduced by FDA in 2018s, where fluoroquinolones use is prohibited for patients suffered from aortic aneurysm because they cause aortic damage [22, 23].

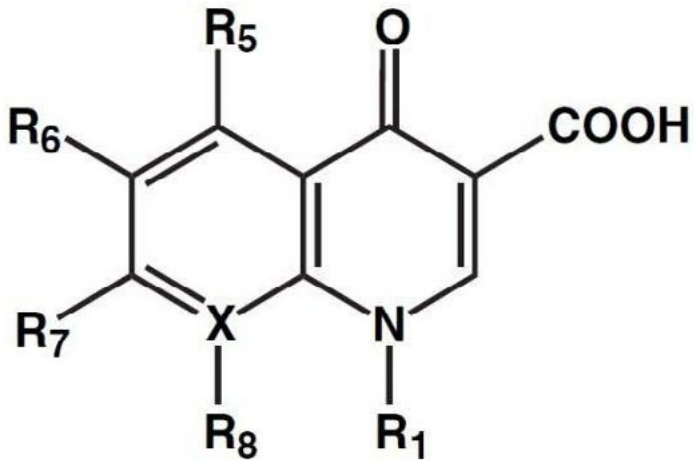

Figure 1: Core structure of quinolone antibiotics

Table 1: Different generations of quinolones and their spectrum activities

Generation	Name	Spectrumactivity
1	Nalidixic acid	Gram-negative bacteria (except Pseudomonas spp.)
2a	Enoxacin, norfloxacin and ciprofloxacin	Gram-negative bacteria, M. pneumonia and C. pneumonia
2b	Ofloxacin and lomefloxacin	Gram-negative bacteria and S. aureus
3	Sparfloxacin, grepafloxacin, clinafloxacin and gatifloxacin	Gram-positive bacteria
4	Moxifloxacin, gemifloxacin, trovafloxacin and garenoxacin	Aerobic and anaerobic Gram-positive and Gram-negative bacteria

The proper selection of fluoroquinolone to complete inhibit the pathogenic bacteria is depending on accurate classification. Four groups of fluoroquinolones are present (Table 2); the first group is only available for oral use and is widely used in the treatment of urinary tract infections and others. For example, norfloxacin is used in the treatment of bacterial enteritis, gonorrhoea, and prostatitis, and pefloxacin is mainly used in the treatment of uncomplicated cystitis. All the members of the second group have *in vitro* potency against Enterobacteriaceae, *Pseudomonas aeruginosa and Haemophilus influenzae*, and they exhibit moderate potency against staphylococci, pneumococci and enterococci; as well as, atypical pathogens like *Chlamydia and Mycoplasma*. All the members of the second group are administered in oral or parenteral form, except enoxacin is administered only in oral form and used in the treatment of urinary tract infections. The second group of fluoroquinolones is used in the treatment of respiratory tract infections caused by Gram-negative bacteria in particular, skin, soft tissue and bone infections; as well as, systemic infections including septicaemia. The third and fourth groups differ from the second one, where the first two have a higher activity against Gram-positive bacteria, such as staphylococci, streptococci, pneumococci and enterococci. Moreover, they have improved activity against atypical pathogens such as *Chlamydia and Mycoplasma*, and in the fourth group also has an improved activity against anaerobes [23].

Table 2: Classification of fluoroquinolones

Group	Description	Members
1st	Oral forms treat urinary tract infections	Norfloxacin and pefloxacin
2nd	Oral and parenteral forms treat systemic indications	Enoxacin, fleroxacin, ofloxacin and ciprofloxacin
3rd	Oral and parenteral forms treat Gram-positive and atypical pathogens.	Levofloxacin, sparfloxacin and grepafloxacin
5th	Oral and parenteral forms treat Gram-positive, atypical pathogens and anaerobes.	Gatifloxacin, trovafloxacin, moxifloxacin and clinafloxacin

Actinobacteria are Gram-positive bacteria which mainly habitat the soil and they are good producers for a wide array of secondary metabolites including vitamins, amino acids, nucleotides, and antimicrobial agents. Quinolones are synthetic-bactericidal agents that inhibit the enzyme topoisomerase II, a DNA gyrase necessary for the replication of the microorganism. Topoisomerase II enzyme produces a negative supercoil on DNA, permitting transcription or replication; thus, by inhibiting this enzyme, DNA replication and transcription are blocked. The polyketides are another class of natural antibiotics synthesized through the decarboxylative condensation of malonyl-CoA-derived extender units in a process similar to the fatty acid synthesis. The polyketide chains produced by a minimal polyketide synthase are often further modified (e.g., glycosylated) into bioactive natural products. This study aims to constrict prevalence and exacerbation of Gram-negative bacteria infections by production of an effective antibacterial compound from *S.griseorubens*. This antibacterial compound will be extracted, purified and characterized to be ready for another study. This work provided a new chemical formula that has very potent antibacterial activity in particular against Gram-negative bacteria. This chemical formula is similar with that of quinolone antibiotic. This chemical formula is naturally produced by actinobacteria and may be chemically modified to introduce a broad spectrum and an effective derivative.

Results

Isolation and screening of actinobacteria

There three soil samples were collected from divergent locations in the farm of National Organization for Drug Control and Research (NODCAR). The soil which are cultivated in particular is still the mother habitat of microorganisms especially actinobacteria, which decompose the organic substances within the soil and provide easy nutrients for plants. Forty unrepeated actinobacterial isolates were isolated on the agar surface of starch nitrate medium. Each isolate has been assigned with a certain code that was divided into a symbol "Eg" and a serial number. The antibacterial activity of actinobacterial isolates was detected by screening against test pathogenic Gram-negative bacteria using agar-disc diffusion method. All clinical researches proved that, Gram-negative bacteria are more deleterious than Gram-positive bacteria due to different reasons including presence of outer envelope and biofilm which retard delivering of antibiotics inside the bacterial cell. Gram-negative bacteria are usually correlated with serious diseases like respiratory system diseases and urogenital tract infections especially if antibiotics-resistance genes are present labeled either on the chromosomes or extra-chromosomes like plasmids or transposons. The screening test resulted in presence of twenty-two isolates which produced different levels of an antibacterial activity. As well known, actinobacteria and *Streptomyces* spp in particular are characterized by a high ability for secondary metabolites production including antimicrobial agents, which are widely used in the medical field. Notably, the levels of an antibacterial activities were relatively similar, except that of the isolate "Eg-7" was has a highest level of an antibacterial activity, so this isolate was selected as a most potent antibacterial producer (Table 3).

Table 3: Antibacterial activities of active actinobacterial isolates

Isolate (Eg)	Antibacterial activity measurement by an inhibition zone (mm)				
	A. lwoffi	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*E. cloacae*
5 & 6	25.0	30.0	25.0	22.0	20.0
7	42.0	45.0	35.0	30.0	45.0
11	20.0	25.0	20.0	25.0	20.0
15	20.0	20.0	25.0	30.0	25.0
16	25.0	27.0	20.0	27.0	25.0
17 & 18	25.0	30.0	20.0	25.0	30.0
20	20.0	20.0	25.0	27.0	20.0
22 & 23	25.0	20.0	25.0	30.0	20.0
25	30.0	25.0	20.0	25.0	25.0

26	30.0	20.0	20.0	20.0	25.0
27	25.0	27.0	20.0	30.0	25.0
30	25.0	27.0	20.0	25.0	20.0
32	25.0	20.0	25.0	25.0	20.0
33	20.0	25.0	25.0	20.0	27.0
34 & 36	20.0	20.0	20.0	20.0	27.0
37	20.0	30.0	25.0	20.0	20.0
38	20.0	25.0	25.0	20.0	30.0
40	20.0	25.0	25.0	27.0	30.0

Identification of actinobacterial isolate

The actinobacterial isolate Eg-7 was identified using classical and gene analysis techniques. According to International *Streptomyces* Project (ISP), six agar media were used to determine the cultural characteristics including growth rate, color of aerial and substrate mycelia, and the color of diffusible pigment (Table 4). The microbiological media of actinobacteria are rich with nutrients and carbon and nitrogen sources in particular, because they are consumed by the bacteria to accomplish the required vital physiological processes including propagation, sporulation, excretion, energy generation, and others. The actinobacterial isolate Eg-7 was found has gray aerial mycelia on the agar surface of all recommended media, while it was found has a yellow substrate mycelium on the agar surface of yeast-malt extract (ISP-2), and brown color on the agar surface of the other media. This isolate was moderately grown on the agar surface of both yeast-malt extract (ISP-2) and inorganic salts starch (ISP-4) media, while a good growth was shown on the agar surface of oatmeal extract (ISP-3) medium, and a poor growth was observed on the agar surface of both peptone yeast extract iron (ISP-6) and tyrosine (ISP-7) media. Although it was found produce a yellow diffusible pigment on the agar surface of both yeast-malt extract (ISP-2) and oatmeal extract (ISP-3) media, it didn't produce diffusible pigments on the agar surface of the other media.

Table 4: Cultural characteristics of an actinobacterial isolate Eg-7

Agar media	Growth	Color		
		AM	SM	DP
Yeast-malt extract ISP-2	Moderate	Gray	Yellow	Yellow
Oatmeal extract ISP-3	Good	Gray	Brown	Yellow
Inorganic salts starch ISP-4	Moderate	Gray	Brown	None
Glycerol asparagine ISP-5	Good	Gray	Brown	None
Peptone yeast extract iron ISP-6	Poor	Gray	Brown	None
Tyrosine ISP-7	Poor	Gray	Brown	None
AM, Aerial Mycelia; SM, Substrate Mycelia, DP, Diffusible Pigment				

Physiological and biochemical characteristics of the actinobacterial isolate Eg-7 have been determined (Table 5). The regular morphology of actinobacteria depends on the age of culture, so it must be harvested and prepared at a proper time to can use by scanning electron microscope. The spiral spore chain was appeared with ellipsoidal smooth spores (Fig. 2). The gray spore mass was appeared, and the brown and yellow colors were shown for substrate mycelia and diffusible pigment, respectively. No motility was recorded for this isolate, and the structure of cell wall was found has LL-diaminopimelic acid, and the sugar pattern didn't be detected. This isolate produced melanoid pigment and hydrogen sulfide. Xanthin and esculin were recalcitrant substances where they didn't degrade. The growth of the isolate Eg-7 was not observed at high concentrations of NaCl (10 – 15%). Streptomycin and amoxicillin antibiotics completely inhibited the growth of the actinobacterial isolate Eg-7. The isolate couldn't grow at high values of temperature (≥ 40°C) and pH (≥ 10). Apart of amylase, protease and catalase which were produced, other enzymes never produced. Apart of L-arabinose which didn't utilize, other sugars were utilized as a carbon source. Finally, all amino acids were utilized as a nitrogen source. The sequence analysis of 16S rRNA gene was performed as a confirmatory identification of the actinobacterial isolate Eg-7. Comparison has been carried out between the sequence of 16S rRNA gene and reference strains of *Streptomyces* sequences using DNA BLASTn (NCBI website). The classical and gene analysis methods proved that, the actinobacterial isolate Eg-7 is belonging to *Streptomyces* genera, and *S. graverobbers* in particular due to a high similarity percentage (99%) between test and reference strains.

Table 5: Physiological characteristics of an actinobacterial isolate Eg-7

Parameter	Test	Result
Morphological characteristics	Shape of spore chain	Spiral
	Spore shape	ellipsoidal
	Spore surface	Smooth
	Motility	Non-motile
Cell wall hydrolysis	Diaminopimelic acid (DAP)	LL-DAP
	Sugar pattern	Not detected
Physiological characteristics	Melanoid and H2Sproduction	Positive
	Xanthin and esculin degradation	Negative
	Tolerance of NaCl (10-15%)	Negative
	Streptomycin and amoxicillin resistance	Negative
	Growth at ≥ 40°C	Negative
	Growth at ≥ 10 pH	Negative
Enzymatic activity	Amylase, protease and catalase production	Positive
	Lipase, cellulase and coagulase production	Negative
	Nitrate reductase and urease production	Negative
Utilization of different carbon sources	Glucose, fructose, galactose and sucrose	Positive
	Raffinose, rhamnose and mannitol	Positive
	Arabinose	Negative
Utilization of different nitrogen sources	Cystiene, valine, alanine, leucine and histidin	Positive
	Lysine, tyrosine and phenylalanine	Positive

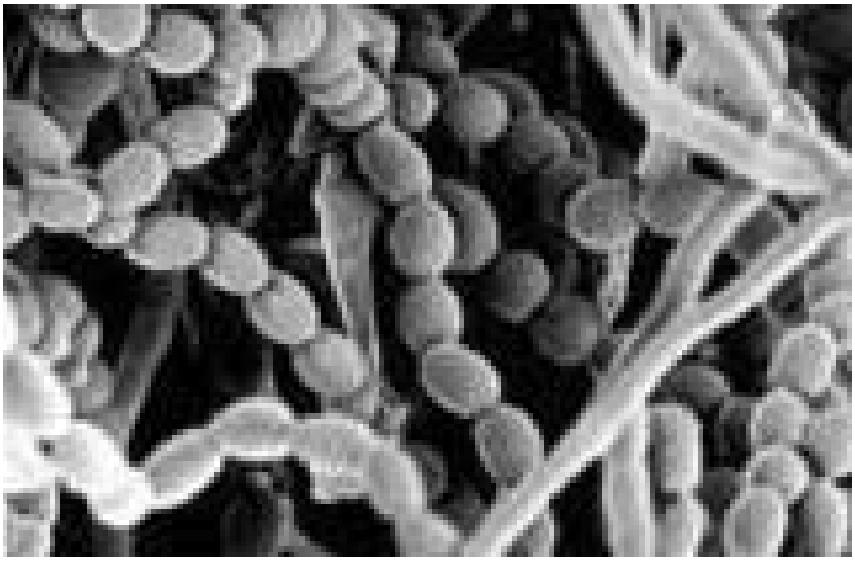

Figure 2: Scanning electron micrograph of the actinobacterial isolate Eg-7

Extraction of antibacterial compound

The antibacterial compound was extracted from the cell free extract of starch nitrate broth medium inoculated with *S. griseorubens* using ethyl acetate. The ethyl acetate extract was separated by HPLC using methanol and water as a mobile phase. The peaks of potential antibacterial compounds were appeared with other peaks of polar and nonpolar impurities (Fig. 3). The last three active peaks were retained at 41.90, 43.43, and 44.54 min, respectively, and all five active peaks were retained between 40 and 45 min. The largest active peak was dried using N2 gas and its solubility was tested using acetonitrile, DMSO, methanol, ethanol, and 2-propanol. Apart of ethanol and 2-propanol, methanol, acetonitrile, and DMSO could solubilize an antibacterial

compound. Fraction containing polar compound displayed more effective antibacterial activity. The solvents affected the extract containing an antibacterial compound, where polar solvent (water) removed all polar impurities, and non-polar solvent (n-pentane) removed all non-polar impurities.

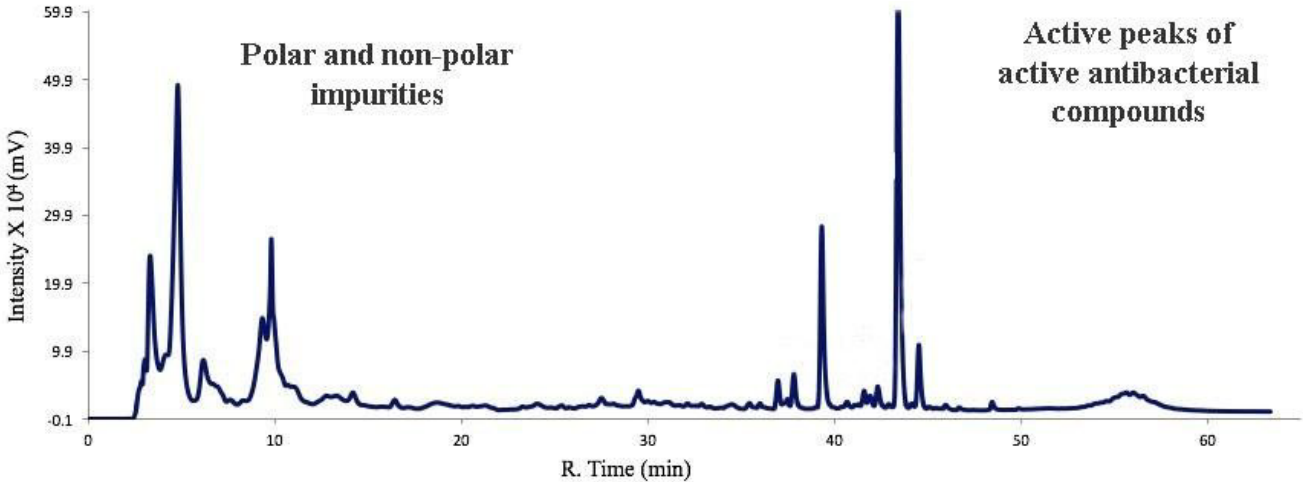

Figure 3: HPLC chromatogram of an extract of *S. griseorubens* in methanol

Purification, mass spectrum and IR analysesof antibacterial compound

The crude extract of an antibacterial compound was analyzed by liquid chromatography mass spectrum (LC-MS), which resulted in numerous peaks, but the active one was displayed at m/z 721.325. This crude extract was exposed to purification process through FCC and GFCC. FCC was perfectly packed with a silica gel 60 as a stationary phase, and DCM/methanol mixture was used as a mobile phase. The elution and fractionation of the crude extract have been carried out, and the antibacterial activity was tested with each fraction. The active fractions were pooled and dried by N_2 gas, and then delivered to GFCC which was packed with sephadex LH-20. The elution of the mixture was carried out by methanol to obtain several fractions. The antibacterial activity was tested with each fraction, where only four active ones were obtained, pooled and dried using N_2 gas. The absorbance of each active fraction was measured at 254 nm, and the broad active peaks were displayed (Fig. 4). The most active fraction was analyzed by IR spectrum, which showed a broad absorption at 3338 cm^{-1} that is characteristic of a hydroxyl group, an absorption at 3079 cm^{-1} associated with an alkene, and the absorption bands around 3000 cm^{-1} that indicated the -CH stretches of alkane groups. Absorption at 1728 cm^{-1} confirms the presence of a carbonyl functional group in the molecule (Fig. 5). The mass spectrum analysis of an antibacterial compound was performed to determine its molecular formula, which was recorded as $C_{35}H_{26}N_6O_4$.

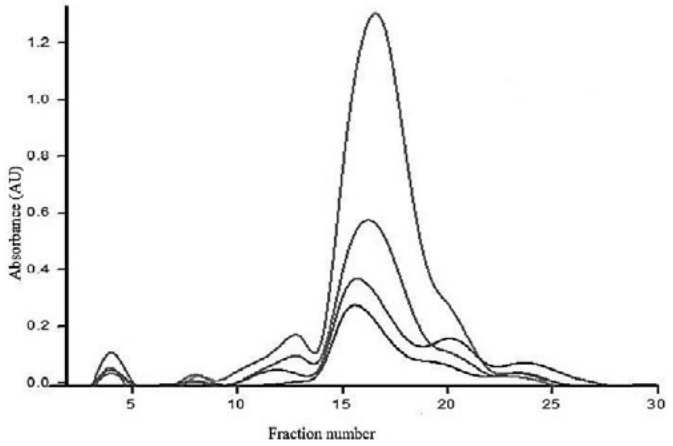

Figure 4: UV spectra of active fractions of sephadex LH-20 column eluted with methanol

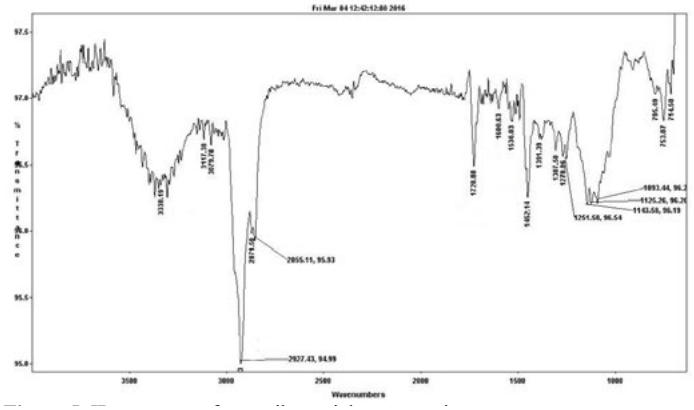

Figure 5: IR spectrum of an antibacterial compound

NMR analysis

Structure determination has been carried out using Bruker BioSpin and Billerica MA for 1D (^{1}H NMR, ^{13}C NMR) and 2D (HSQC, COSY, TOCSY, and HMBC) extensive NMR spectral analysis. Structure determination of an unknown compound using homonuclear 1H-1H COSY depends on the ability to detect couplings between neighboring protons. Correlations between protons and neighboring carbons can be detected using the heteronuclear HMBC spectrum. Based on the characteristic UV-Vis absorption maxima at 277 and 327 nm; strong IR absorptions at 3338 cm^{-1} (-OH), 3079 cm^{-1} (=CH-); large molecular weight 721.325 Da with a higher number of carbon, hydrogen, and oxygen atoms from HR-MS and proton signals from ^{1}H NMR, the compound of interest would most likely be a polyketide. Close inspection of spectral data revealed the presence of minor peaks in NMR spectra, which could be related to the decomposition of the natural product during the purification process, as well as distinctive tautomers or conformers. Apart from minor signals, the structure analysis of the unknown polyketide was started with major peaks present in CD_3OD. ^{1}H (Fig. 6) and ^{13}C (Fig. 7) NMR data in combination with the HSQC analysis showed the presence of 35 carbons attributable to seven (7) sp^3 methyl (-CH_3) groups among which one was an oxygenated methyl carbon. There are eleven carbons corresponded to sp^2 methine (-CH=C), ten carbons sp^3 methylene (-CH_2-), eighteen carbons are sp^3 methine, and six carbons are quaternary carbons. ^{13}C-NMR interpretation disclosed three sets of distinct chemical shifts; saturated methyl (-CH_3), methylene (-CH2), and methine (sp^3 CH).

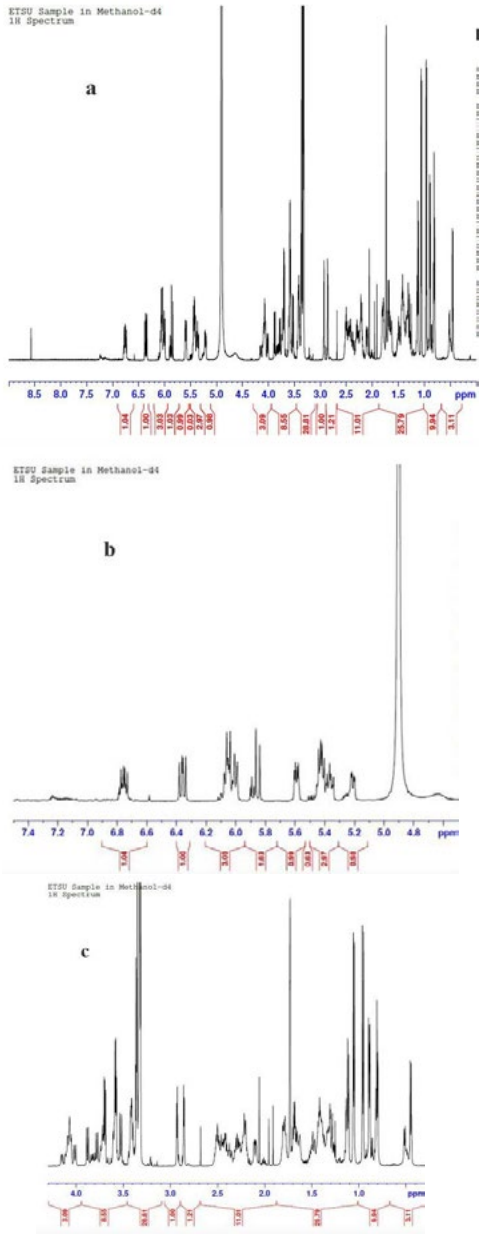

Figure 6: ^{1}H NMR spectra of an antibacterial compound, (A) C1, (B) C2, (C) C3

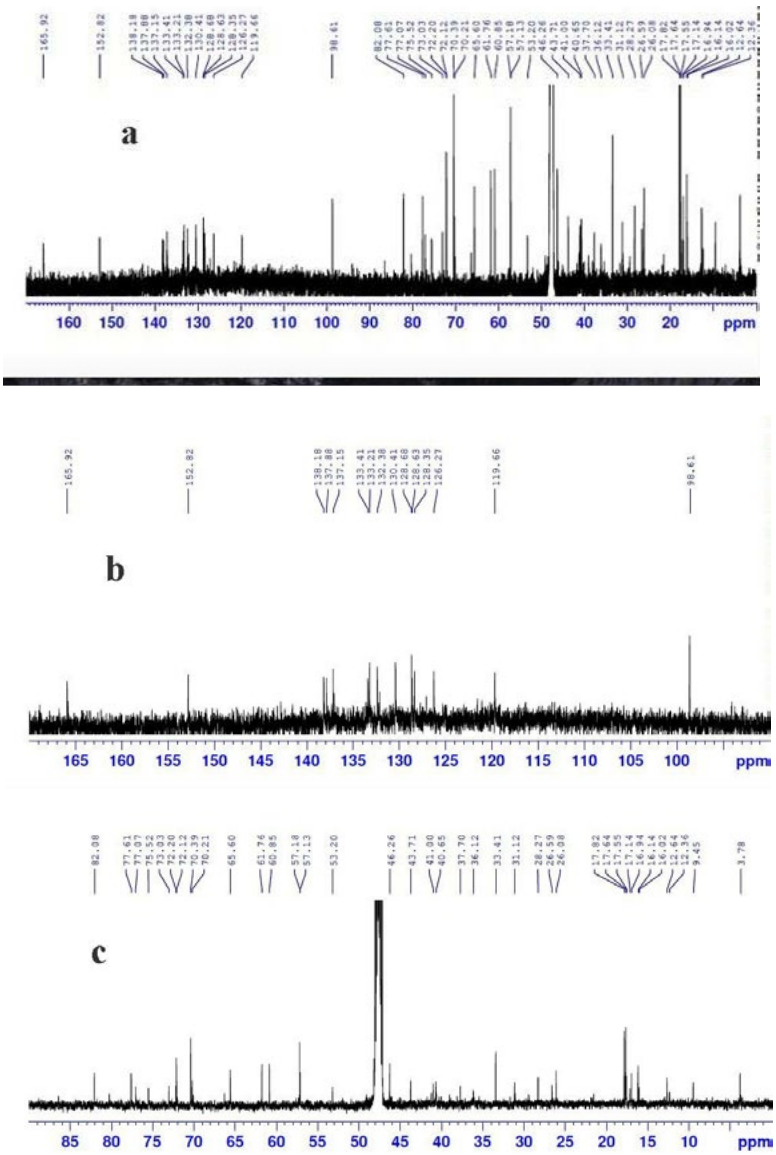

Figure 7: ^{13}C NMR spectra of an antibacterial compound, (A) D1, (B) D2, (C) D3

Discussion

Streptomycetes can survive in a wide array of environments including the soil, water, air, wastes, and even on the solid surfaces. Moreover, they can live under mild and extreme conditions such as hot, cold and salty environments. This unique ability of actinobacteria is due to various factors including sporulation profile, utilization of a wide array of carbon sources and production of antimicrobial agents. In addition, a broad metabolic profile of streptomycetes is due to presence of large genome containing hundreds of transcription factors that control gene expression, allowing them to respond to specific needs [24]. The most potent antibiotic producer is *Streptomyces* genus so far. Apart of antibiotics, different bioactive compounds were produced by *Streptomyces* and utilized as effective pharmaceutical products including anticancer and immunosuppressant compounds [25].

The most potent antifungal actinobacterial isolate was identified as *S. griseorubens* using phenotypic and molecular methods. The morphology was found appear as branched substrate and aerial mycelia which carried smooth-surfaced ellipsoidal spores in open hooked spore chains. The cell wall contained LL-diaminopimelic acid and the sugar pattern didn't detect. A grayish aerial spore mass was appeared on 7 standard media recommended for the genus *Streptomyces*. The identification was confirmed by analysis of 16S rRNA gene sequence, which was found has 1096bp [26].

1-butanol was the first-choice organic solvent for extraction of an antibacterial compound from the filtrate of *Rhodococcus* sp. by HPLC due to low solubility and immiscibility in an aqueous solution. Therefore, 1-butanol is considered one of effective alcoholic solvents used for an extraction of antimicrobial compounds. The antibacterial compound was extracted by 1-butanol from both wild and mutant strains, and the antibacterial activity of extracted antibacterial compound from mutant strain was tested using agar-disc diffusion method compared with that of wild strain. UV-Vis spectroscopy analysis was carried out before fractionation process [27]. According

to the analysis of IR spectrum of an antibacterial compound that is identified in the later as quinolone, the active peaks elucidated at the range 1735–1707 cm-1 due to the vibration of carboxylic group, while the intense one elucidated at 1617 cm^{-1} which assigned as *ν*(C=O)*p*, where p is pyridone. The peak which displayed at 3400–3200 cm^{-1} assigned to O–H stretching vibrations. Two characteristic peaks at 1556 and 1494 cm^{-1} that could be assigned as m(O–C–O) asymmetric and symmetric stretching vibrations; respectively, moreover, it is very difficult to assign other vibrations in the spectra of ciprofloxacin due to the presence of other numerous peaks could be present in these regions such as the presence of C=C stretching vibration of the aromatic rings around 1620 cm^{-1} and the CH bending vibrations (m-CH) in the region between 1440 and 1550 cm^{-1} and the stretching vibration of the quinolone ring system (m ring) around 1400 cm^{-1}[28]. The mass spectrum of quinolone was performed using ESIMS/MS technique which was used in characterization of several compounds. The signal appears at 331 m/z refers to appearance of the molecular formula $[C_{17}H_{19}FN_3O_3]$+ which is high stable [29]. The absorption spectrum of quinolone was determined at 0.25 × 10-5 mol/L in ethanol. The characteristic absorption peak was displayed at 322 nm which could be due to n–p* (HOMO–LUMO) electronic transition. The second active peak with high absorbance was displayed at 275 nm. Unsaturated hydrocarbons attached to O_2 atom are characterized by these transitions [30]. The internal hydrogen atoms of quinolone structure link between the hydroxyl residue of the carboxyl group and the carbonyl residue of the pyridone nucleus to affect the chemical shifts of C-4 and CO signals, which are shifted downfield. The implementation of the substituent on nitrogen changed the carbon chemical shift in the 2position. For all carbons of the benzene ring, incremental chemical shifts elicited by the presence of the 3substituted 4-quinolone ring, therefore similar data pertinent to the fused triazole or pyrazine ring, allow the prediction of chemical shifts in various fused multi-ring systems [31].

Methods

Sampling of soils and isolation of actinobacteria

Three soil samples were collected from divergent locations in the farm of National Organization for Drug Control and research (NODCAR). The soil sampling was accomplished at depth 10 cm to avoid the arid surface that is usually free from microorganisms. The soil sample was directly placed in a sterile plastic bag and fast transported to the laboratory for air drying. According to Johnson et al.[32], 10 g of air-dried soil were suspended in 100 ml of sterile distilled water for 20 min to make a soil suspension, which has been filtered through sterile cotton piece to obtain a semi-clear soil extract. Soil extract was diluted using serial dilution method (10^{-1} to 10^{-8}). One ml was withdrawn from each fraction, and then pipetted and streaked by sterile glass rod on the agar surface of starch nitrate agar medium33.All plates were incubated at inverted position for 7 days at 30°C. After incubation period and by visual examination, all separated actinobacterial colonies were picked up independently by a sterile platinum loop, and then streaked on the agar surface of starch nitrate agar medium to obtain purified actinobacterial isolates, which were preserved on agar surface of starch nitrate slants at 4°C for one month and subcultured regularly. Duplication of plates has been performed.

Test pathogenic bacteria

American Type Culture Collection Laboratory (ATCC) was the sole source of test pathogenic Gram-negative bacteria. Five strains were obtained named *Acinetobacter lwoffi* ATCC 17925, *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 33495, *P. aeruginosa* ATCC 35032, and *Enterobacter cloacae* ATCC 35030.

Antibacterial test

The antibacterial activities of actinobacterial isolates were detected by screening them against pathogenic Gram-negative bacteria. The pathogenic bacterial strains were prepared by culturing them in soybeancasein dextrose broth medium, which has been incubated for 24 h at 35 ± 2°C. Actinobacterial discs of 7 days old were placed on the agar surface of seeded soybean-casein dextrose agar plates with pathogenic bacterial strains, and all plates were incubated for 24 h at 30°C. Examination of plates has been carried out visually to determine the antibacterial activities represented by appearance of an inhibition zones around active actinobacterial discs.

Identification of the most potent antibacterial isolate of actinobacteria

Identification of the most potent antibacterial isolate of actinobacteria was performed according to Shirling and Gottlieb[34], where morphological, cultural and physiological characteristics were determined. Confirmatory identification was carried out by analysis of 16S rRNA gene sequence. The DNA was extracted according to Sambrook et al.[35], and the concerned gene was amplified by a thermocycler (Cetus Model 480; PerkinElmer, Waltham, MA, USA) using the universal primers 27f (5′-AGA GTT TGATCC TGG CTC AG -3′) and 1525r (5′-AAG GAG GTG ATC CAG CC-3′) at 94°C for 5 min, 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 90 s, and a final extension step at 72°C for 5 min. The product was sequenced by a BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) on an ABI 310 automated DNA sequencer (Applied Biosystems). Homology of the 16S rRNA sequence was analyzed using the BLAST algorithm that is an available in GenBank.

Extraction of an antibacterial agent

Starch nitrate broth (1500 ml) was prepared and distributed into 3 flasks (1 L), where each one was contained 500 ml. Each flask was inoculated with one ml of actinobacterial suspension (most potent). All flasks were incubated in a shaking incubator at 20°C and 120 rpm for 14 days. After incubation period, the actinobacterial suspensions of three flasks were pooled and supplemented with 300 ml of 1-butanol because it is an immiscible with an aqueous solution. This mixture was incubated in a shaking-incubator at 20°C and 120 rpm for 1 h. After incubation period, the actinobacterial suspension was centrifuged at 6000 rpm for 10 min, and the supernatant was separated using a separating funnel, the top organic layer (1-butanol) was collected into a beaker, and both layers (organic and the aqueous) tested for activity to ensure the all of the compounds had been extracted from the broth culture. The butanol extract was evaporated using a rotor evaporator at 25°C [36].

Antibacterial activity test

The antibacterial activity of an extract was tested against pathogenic Gram-negative bacteria using agar-disc diffusion method [37]. A loopful of a single bacterial colony was immersed in 2 mL of soybean-casein dextrose broth. All test tubes were incubated in water bath with shaking at 27°C for 18 h. The sterile filter paper disk was saturated with an extract and placed on an agar surface of bacterial seeded plates, which were incubated for 24 h at 35 ± 2°C. Appearance of an inhibition zone around the disk refers to an antibacterial activity and vice versa.

Flash column chromatography (FCC)

The first separation step of an antibacterial agent was performed by FCC that was packed with a silica gel 60 as a stationary phase [38, 39]. The separation by FCC depends on the partitioning between silica gel 60 (stationary phase) and dichloromethane (DCM) (mobile phase), which migrated under low pressure. Different compounds including an antibacterial compound in the crude mixture have different affinities with the stationary phase due to a chemical charge or an adsorption operability [40, 41]. FCC was sealed at the bottom with a piece of cotton-wool and a small layer of sand (1-2 cm), and then the silica gel 60 was gently poured until successful packing has been accomplished. Vacuolation was done through the stopcock of the column to condense the silica gel and to obtain a tight packing. Sodium sulfate solution was poured on the top of the column to prevent charges exhaustion. DCM was gently poured with vacuolation onto the column until solvents elution carried out. The dried butanol extract was mixed with silica gel 60 and a low amount of DCM. This mixture was passed through a stationary phase with methanol as polar solvent and DCM as non-polar solvent. Different ratios of DCM and methanol (v/v) were elucidated as shown in (Table 6). The fractionation has been carried out (10 mL/fraction), and the absorbance of each one was measured at 254 nm by using UV-spectrophotometer. The antibacterial activity was detected for each fraction by using the agar-disk diffusion method. The active fractions were pooled and let stand under fume hood to evaporate the solvent and to dry the mixture, which was dissolved in 2-propanol and stored at 4°C for further purification.

Table 6: DCM and methanol ratios used in flash column chromatography

DCM (volume in ml)	Methanol (volume in ml)	DCM-Methanol ratio
300	0	1:0
300	10	30:1
300	20	15:1
300	30	10:1
250	50	5:1
150	150	1:1
0	300	0:1

Gel filtration column chromatography (GFCC)

The mixture of FCC was transmitted to GFCC, which depends on the size of molecules in separation process. The large molecules never face any retardation during their trip of migration through stationary phase, because the big size of them don't allow to enter the pores of stationary phase and therefore they move easily away the resin under pressure of mobile phase. On contrary, the small size of some molecules enables them to enter inside the molecules of the resin through the pores, and therefore they be retarded and eluted slowly, so small molecules can be picked up at the last fractions. Sephadex™ LH-20 was used as a resin of the column, and it was prepared by hydroxypropylation of sephadex G-25, which has hydrophilic and lipophilic characteristics, so it swells in aqueous solutions and organic solvents. Sephadex™ LH-20 dry powder (28.78 g) was placed in a beaker (250 ml), and supplemented with methanol gradually with gently stirring until be swollen during 2 h. The beaker was shaken every 30 min to remove any air bubbles trapped in the medium. The sephadex was poured gently inside the glass column chromatography where the column was filled evenly without forming of air bubbles. The solvent was added and passed through the resin under atmospheric pressure. The solvent was withdrawn through the opened stopcock of the column until the column was tightly packed, and then the stopcock was closed. The dried 1-butanol extract was dissolved in a low amount of isopropanol and passed through a sephadex and eluted with methanol as a mobile phase. The fractions were collected and tested for antibacterial activity. The absorbance of each fraction was measured by UV-spectrophotometer at 254 nm. The active fractions were pooled and let stand under fume hood to be dried. The dried extract was dissolved in a low amount of 2propanol to be prepared for high-performance liquid chromatography (HPLC) analysis [42].

HPLC analysis

The antibacterial extract was purified by HPLC (LC-10AS), which was equipped with 2 solvents; A and B. Two pumps A and B were used to pump the mobile phase to generate a maximum pressure of 6000 psi (lb/in.2) or 414 bar [43, 44]. Mobile phase degassing inside the pump was mainly used to prevent formation of the air bubbles, which cause a problem in the solvent delivery and forms specious peaks in the output by the detector [45]. The solvents were placed inside HPLC with a stir bar, which was connected to a vacuum pump by the stopper. HPLC was equipped with UV-Vis detector (SPD-10A) [46].

Characterization of an antibacterial agent

The antibacterial compound was characterized according to

Butler47. Stepwise of characterization has been achieved to detect and elucidate the identity of an antibacterial compound. UV-Vis spectroscopy, IR spectroscopy, mass spectroscopy, and NMR (1D and 2D) were used as urgent tools for chemical formula elucidation.

Ultraviolet-Visible Spectroscopy (UV-Vis)

UV-Spectra (280 – 400 nm) of all fractions of both FCC and GFCC were measured by UV-Vis spectrophotometer (Carey 8454), and methanol was used as a blank. The purified compound of HPLC was dissolved in acetonitrile and its absorbance was measured at 254 nm, and acetonitrile was used as a blank [47].

Infrared spectroscopy (IR)

IR-spectra were measured in KBr pellets with a spectral range of 6,000-350 cm-1 using a genesis II FTIR spectrometer.

Liquid chromatography-mass spectroscopy (LC-MS)

The crude extract of the most potent actinobacterial isolate was exposed to LC-MS analysis. The analysis was done using the gradient elution method with solvents A and B containing 0.1% aqueous formic acid and acetonitrile, respectively. The program started with 30% B which was increased to 40% B from 0-5 min. After 5 to 10 min it was increased to 50% B; from 10 to 15 min it increased to 60% B; from 15 to 20 min it increased to 80% B; from 20 to 25 min increased to 100% B, which was maintained from 25 to 35 min, at a flow rate 1.0 ml/min. From 35 to 35.5 min 100% B was dramatically decreased back to 30% B, which was kept from 35.5 to 40 min, to reconstitute the column before the next run. The column oven temperature was maintained at 25°C, and the temperature of the autosampler was set to 4°C. An electrospray ionization probe was used to ionize the sample at a 3.5 kV spray voltage. The sheath gas flow was set to 50 units and the auxiliary gas was set to 25 units. The S-lens level was set to 50 units. The conditions were kept constant for positive ionization mode acquisition. For complete scan profiling experiments, the MS was run with a resolution of 140,000 and with a scan range of 80-1000 m/z, for all ion fragmentation (AIF) scans. The resolution was 140,000 with a scan range of 80-1000 m/z, with a normalized collision energy (NCE) of 20 eV. Xcalibur software was used to examine the results [47].

High-resolution mass spectrometry (MS)

The high-resolution mass analysis was carried out on Bruker maXis II mass spectrometer. The sample was ionized using electrospray ionization in positive (ESI+) mode. The sample was dissolved in an acetonitrile and water mixture (50:50) with 0.1% formic acid.

Nuclear magnetic resonance (NMR) spectroscopy

^{1}H-NMR, ^{13}C-NMR, and 2D-NMR experiments were performed on a Bruker Avance II 600 MHz NMR spectrometer (^{1}H 600 MHz; ^{13}C 150 MHz). It was equipped with 5 mm probe using deuterated methanol as a solvent. All NMR experiments were carried out at room temperature. Chemical shift values were measured in parts per million (δ, ppm). The coupling constants value (J) was described in Hz. The splitting patterns of proton signals were also designated as follows: singlet (s), doublet (d), a doublet of doublets (dd), a doublet of the doublet of doublets (ddd), triplet (t), the quartet (q), and the multiplet (m).

Acknowledgements

Authors sincerely thank Head and members of Microbiology Department for their support to this work, and also deeply grateful to Head and members of Biology Department for providing the necessary support to can complete this work.

Author contributions statement

W. M. provided the scientific idea, and performed collection of soil samples, isolation and identification of actinobacterial isolate, antibacterial test, and writing and correspondence of a manuscript for publication. M. A. brought-in the bacterial strains, and performed extraction, purification and characterization of an antibacterial compound.

Additional information

Correspondence and requests for materials should be addressed to Waleed M. Abdulkhair. The corresponding author is responsible for submitting a competing interest's statement on behalf of all authors of the paper.

References

1. Bisacchi, G. S. (2015). Origins of the quinolone class of antibacterials: an expanded "discovery story" miniperspective. Journal of medicinal chemistry, 58(12), 4874-4882.
2. Liu, H., & Mulholland, S. G. (2005). Appropriate antibiotic treatment of genitourinary infections in hospitalized patients. The American journal of medicine, 118(7), 14-20.
3. Lesher, G. Y., Froelich, E. J., Gruett, M. D., Bailey, J. H., & Brundage, R. P. (1962). 1, 8-Naphthyridine derivatives. A new class of chemotherapeutic agents. Journal of Medicinal Chemistry, 5(5), 1063-1065.
4. Emmerson, A. M., & Jones, A. M. (2003). The quinolones: decades of development and use. Journal of Antimicrobial Chemotherapy, 51(suppl_1), 13-20.
5. Adjei, M. D., Deck, J., Heinze, T. M., Freeman, J. P., Williams, A. J., & Sutherland, J. B. (2007). Identification of metabolites produced from N-phenylpiperazine by Mycobacterium spp. Journal of Industrial Microbiology and Biotechnology, 34(3), 219-224.
6. Andersson, M. I., & MacGowan, A. P. (2003). Development of the quinolones. Journal of Antimicrobial Chemotherapy, 51(suppl_1), 1-11.
7. Mitscher, L. A. (2005). Bacterial topoisomerase inhibitors: quinolone and pyridone antibacterial agents. Chemical reviews, 105(2), 559-592.
8. Anderson, V. E., & Osheroff, N. (2001). Type II topoisomerases as targets for quinolone antibacterials turning Dr. Jekyll into Mr. Hyde. Current pharmaceutical design, 7(5), 337-353.
9. Lima, C. D., & Mondragón, A. (1994). Mechanism of type II DNA topoisomerases: a tale of two gates. Structure, 2(6), 559-560.

10. Hooper, D. C. (2001). Emerging mechanisms of fluoroquinolone resistance. Emerging infectious diseases, 7(2), 337.
11. Aldred, K. J., McPherson, S. A., TurnboughJr, C. L., Kerns, R. J., &Osheroff, N. (2013). Topoisomerase IV-quinolone interactions are mediated through a water-metal ion bridge: mechanistic basis of quinolone resistance. Nucleic acids research, 41(8), 4628-4639.
12. Hooper, D. C., & Jacoby, G. A. (2015). Mechanisms of drug resistance: quinolone resistance. Annals of the New York academy of sciences, 1354(1), 12-31.
13. Sharma, P. C., Jain, A., & Jain, S. (2009). Fluoroquinolone antibacterials: a review on chemistry, microbiology and therapeutic prospects. Acta Pol Pharm, 66(6), 587-604.
14. Van Caekenberghe, D. L., & Pattyn, S. R. (1984). In vitro activity of ciprofloxacin compared with those of other new fluorinated piperazinyl-substituted quinoline derivatives. Antimicrobial agents and chemotherapy, 25(4), 518-521.
15. Abdulkhair, W., & Alghuthaymi, M. (2020). Production and characterization of semi-quinolone antibiotic produced by Streptomyces griseorubens.
16. Van Bambeke, F., Michot, J. M., Van Eldere, J., & Tulkens, P. M. (2005). Quinolones in 2005: an update. Clinical Microbiology and infection, 11(4), 256-280.
17. Abdulkhair, W., & Alghuthaymi, M. (2020). Production and characterization of semi-quinolone antibiotic produced by Streptomyces griseorubens.
18. Forrest, A. L. A. N., Nix, D. E., Ballow, C. H., Goss, T. F., Birmingham, M. C., & Schentag, J. J. (1993). Pharmacodynamics of intravenous ciprofloxacin in seriously ill patients. Antimicrobial agents and chemotherapy, 37(5), 1073-1081.
19. Preston, S. L., Drusano, G. L., Berman, A. L., Fowler, C. L., Chow, A. T., Dornseif, B., ... & Corrado, M. (1998). Pharmacodynamics of levofloxacin: a new paradigm for early clinical trials. Jama, 279(2), 125-129.
20. Croisier, D., Etienne, M., Bergoin, E., Charles, P. E., Lequeu, C., Piroth, L., ... & Chavanet, P. (2004). Mutant selection window in levofloxacin and moxifloxacin treatments of experimental pneumococcal pneumonia in a rabbit model of human therapy. Antimicrobial Agents and Chemotherapy, 48(5), 1699-1707.
21. Perrone, C. E., Takahashi, K. C., & Williams, G. M. (2002). Inhibition of human topoisomerase IIα by fluoroquinolones and ultraviolet A irradiation. Toxicological Sciences, 69(1), 16-22.
22. Singh, S., & Nautiyal, A. (2017). Aortic dissection and aortic aneurysms associated with fluoroquinolones: a systematic review and meta-analysis. The American journal of medicine, 130(12), 1449-1457.
23. Naber, K. G., & Adam, D. (1998). Classification of fluoroquinolones. International journal of antimicrobial agents, 10(4), 255-257.
24. Santhanam, R., Okoro, C. K., Rong, X., Huang, Y., Bull, A. T., Weon, H. Y., ... & Goodfellow, M. (2012). Streptomyces atacamensis sp. nov., isolated from an extreme hyper-arid soil of the Atacama Desert, Chile. International journal of systematic and evolutionary microbiology, 62(Pt_11), 2680-2684.
25. Procópio, R. E. D. L., Silva, I. R. D., Martins, M. K., Azevedo, J. L. D., & Araújo, J. M. D. (2012). Antibiotics produced by Streptomyces. Brazilian Journal of Infectious Diseases, 16(5), 466-471.
26. Al-Askar, A. A., Abdulkhair, W. M., Rashad, Y. M., Hafez, E. E., Ghoneem, K. M., & Baka, Z. A. (2014). Streptomyces griseorubens E44G: a potent antagonist isolated from soil in Saudi Arabia. J Pure Appl Microbiol, 8, 221-230.
27. Couillerot, O., Loqman, S., Toribio, A., Hubert, J., Gandner, L., Nuzillard, J. M., ... & Renault, J. H. (2014). Purification of antibiotics from the biocontrol agent Streptomyces anulatus S37 by centrifugal partition chromatography. Journal of Chromatography B, 944, 30-34.
28. Milata, V., Ilavský, D., Goljer, I., & Zalibera, L. (1994). Thermal cyclocondensation of ethyl (1-methyl-5-and 6-benzimidazolyl/benzotriazolyl) aminomethylenepropanedioates. Collection of Czechoslovak chemical communications, 59(5), 1145-1152.
29. Zayed, M., Nour El-Dien, F., Hawash, M., & Fahmey, M. (2010). Mass spectra of gliclazide drug at various ion sources temperature: Its thermal behavior and molecular orbital calculations. Journal of thermal analysis and calorimetry, 102(1), 305-312.
30. 송창화, 유형원, 박진규, &고동성. (1999). Mechanism of DNA Gyrase Inhibition by Quinolones: Spectral Analysis for Nalidixic Acid Polymorphism. Bulletin of the Korean Chemical Society, 20(6), 727-730.
31. Milata, V., Ilavsky, D., Chudik, M., Zalibera, Ľ., Leško, J., Seman, M., & Belicova, A. (1995). Gould-Jacobs reaction of 6-amino-2, 3-diphenylquinoxaline. Monatshefte für Chemie/Chemical Monthly, 126(12), 1349-1358.
32. Johnson, L. F., & Curl, E. A. (1972). Methods for research on the ecology of soil-borne plant pathogens. Methods for research on the Ecology of Soil-Borne Plant Pathogens.
33. Waksman, S. A. (1961). The Actinomycetes. Vol. II. Classification, identification and descriptions of genera and species. The Actinomycetes. Vol. II. Classification, identification and descriptions of genera and species.
34. Islam, A., & Hernández, F. L. (1966). Methods for characterization of Streptomyces species. METHODS, 16(3), 313-340.
35. Abdulkhair, W., & Alghuthaymi, M. (2020). Production and characterization of semi-quinolone antibiotic produced by Streptomyces griseorubens.
36. Ward, A. L. (2015). Identification of genes required to synthesize an antibiotic-like compound from the soil bacterium Rhodococcus sp. MTM3W5. 2 (Doctoral dissertation, East Tennessee State University).
37. Furtado, G. L., & Medeiros, A. A. (1980). Single-disk diffusion testing (Kirby-Bauer) of susceptibility of Proteus mirabilis to chloramphenicol: significance of the intermediate category. Journal of Clinical Microbiology, 12(4), 550-553.
38. Roge, A. B., Firke, S. N., Kawade, R. M., Sarje, S. K., & Vadvalkar, S. M. (2011). Brief review on: Flash

chromatography. International Journal of Pharmaceutical Sciences and Research, 2(8), 1930.
39. Swathi, G., Srividya, A., Ajitha, A., & Uma Maheswar Rao, V. (2015). Review on: flash chromatography. World J Pharm Sci, 4, 281-296.
40. Abdulkhair, W., & Alghuthaymi, M. (2020). Production and characterization of semi-quinolone antibiotic produced by Streptomyces griseorubens.
41. Andrews, P. (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochemical Journal, 91(2), 222.
42. Hamilton, R. J., & Sewell, P. A. (1982). Introduction to high performance liquid chromatography. In Introduction to high performance liquid chromatography (pp. 1-12). Springer, Dordrecht.
43. Abdulkhair, W., & Alghuthaymi, M. (2020). Production and characterization of semi-quinolone antibiotic produced by Streptomyces griseorubens.
44. Snyder, L. R., Kirkland, J. J. & Dolan, W. J. Introduction to Modern Liquid Chromatography (ed. Wiley) 960 (Washington D. C., 2011).
45. Cunico, R. L., Gooding, K. M., & Wehr, T. (1998). Basic HPLC and CE of Biomolecules. In Basic HPLC and CE of biomolecules (pp. 388-388).
46. Butler, M. S. (2004). The role of natural product chemistry in drug discovery. Journal of natural products, 67(12), 2141-2153.