Original Research Article
Antibacterial Screening and Analysis of Streptomyces coelicolor Secondary Metabolites

Abstract

Aims: The widespread usage of commercially accessible antibacterial agents has resulted in the emergence of multidrug-resistant bacterial pathogens across the world. Since Streptomycetes produce a variety of antibacterial secondary metabolites, the aim of this study was to look into the antibacterial potential of *Streptomyces coelicolor* against some pathogenic bacteria.

Study design: The bacterial strains of S. coelicolor by conducting a study of phenotyping and genotyping for these strains, then extracting the crude extract and actinorhodin using Thin Layer Chromatography technique, and antibacterial assessments.

Place and Duration of Study: Department Medical Microbiology, Science and Health research center, University of Koya, between April 2009 and December 2020.

Methodology: All genomic DNA of strains were extracted and genotypic confirmed by using specific primers, then extracted of crude extract and purified actinorhodin by Thin Layer Chromatography (TLC) and ethyl acetate of Trypticase soy agar plates contained *S. coelicolor*. Crude extract and purified actinorhodin were tested against standard strains of *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 19165), *E. coli* (ATCC 25218), and *Salmonella typhi* (ATCC 14028), also disc diffusion and minimum inhibitory concentration (MIC) techniques were used to serve this purpose.

Results: The crude extract and actinorhodin have an effect as an inhibitor against *S. pyogenes* & *S. aureus* that rang between (10-27 mm), (9-24 mm), while less effect against *E. coli* & S. typhi. The crude extract and actinorhodin exhibited potent activity against *S. aureus & S. pyogenes* that rang MIC values of (8-32µg/ml), while it showed less activity against S. typhi & E. coli with rang of (64-128 µg/ml). The mean growth inhibition values were most significant with (P=.05) outcome.

Conclusion: According to the findings of this study, the extracts be a viable option for generating novel antibiotics to eliminate gram-positive pathogenic bacteria.

Keywords: [crude extract; Streptomyces coelicolor; TLC; actinorhodin; MIC]

1. Introduction

The *Streptomycetaceae* family which belongs to the *Actinobacteria* phylum has shown to be a great source of bioactive small molecules and medicines [1], it is the most abundant drug-producing family in its phylum [2]. In this family, the *Streptomyces* genus has

demonstrated a remarkable ability to produce secondary metabolites, especially antibiotics that have therapeutic potential in humans. Its species parvome has been linked to a variety of biological activities such as inhibiting other microorganisms' growth and/ or killing them [3]. Among these species, the model organism *S. coelicolor* has attracted the interest of researchers due to its ability to produce 20 secondary metabolites, 30% of the compounds belongs to one of the well characterised natural substance. The remaining 70% that called cryptic compounds are not produced in laboratory conditions [4]. *Streptomyces* goes through a cellular development process that is similar to that of fungi [5]. Their development begins with spores that germinate and evolve into a vegetative mycelium of branching hyphae. Most secondary metabolites are generated during the subsequent formation of aerial hyphae, which is thought to be a cell response to nutrition deficiency [6].

Most of the recognized antibiotics in today's arsenal were discovered between 1940 and 1980 including tetracyclines, chloramphenicol, vancomycin, daptomycin, and many more [7]. These chemical signalling molecules/ antibiotics were identified through huge screens of tens of thousands of *Streptomyces* strains undertaken either in academia or industrial [8-9]. The majority of this research was done on culture supernatants to see if they might suppress the development of pathogenic bacteria such as *Staphylococcus aureus* and *Mycobacterium tuberculosis*.

The emergence of multi-drug resistance among human pathogens has pushed the mining for novel antibiotics from existent well-known ancient microorganisms which is *Streptomyces* [10]. *S. coelicolor* can serve this purpose based on its ability to produce two different pigmented secondary metabolites which are actinorhodin with blue pigment and undecylprodigiosin with red pigment in addition to a wide variety of therapeutic compounds such as anti-viral, anti-cancer, immune sensitive modulators, herbicides, insecticides, and anti-parasitic [11-12-13].

The production of this individual derivative is encoded by a cluster with one or more transcription-regulating genes, in the case of actinorhodin (ACT) biosynthesis, AtrA has been identified as ACT activator beside several other proposed transcription factors that can bind to the *act*II-ORF4 promoter region and regulate ACT biosynthesis [14]. Since the most promising types of antibiotics in the future tend to be microbial natural metabolites, the main goal of this work is to investigate the antimicrobial activity of secondary metabolite extracted from *S. coelicolor* strain L646 that has an extra copy of the global regulon *atrA* against some pathogenic bacteria include *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 19165), *Escherichia coli* (ATCC 25218) and *Salmonella typhi* (ATCC 14028).

2. Materials and Methods

2.1 Bacterial strains and culture conditions

Bacterial strains that were used in this study are shown in Table 1. *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* that were used for susceptibility testing were part of a culture collection belonging to the Faculty of Science and Health Research Centre (FSHRC) at Koya University. *Streptomyces* strains were kindly provided by Dr Kenneth MacDowall (University of Leeds/ UK). The bacterial strains, unless otherwise stated, were cultured in/ on Mueller Hinton broth/ agar (MHB or MHA) (Oxoid Ltd, Cambridge, UK) for 24 h at 37°C for antibiotic sensitivity and antibacterial activity tests. All *S. coelicolor* strains were cultured in a 2XYT medium, incubated at 30°C for 6 days [15]. Always *Streptomyces* strains broth cultures were incubated with shaking at 220 rpm in 250 mL Erlenmeyer flasks containing 50 ml of media and fitted with a spring baffle to aid dispersed growth of the mycelia.

Table 1: Bacterial strains used in this study

Strain	Description	Reference/ Source
Staphylococcus aureus	ATCC: 25923	FSHRC collection
Streptococcus pyogenes	ATCC: 19165	FSHRC collection
Escherichia coli	ATCC: 25218	FSHRC collection
Salmonella typhi	ATCC: 14 028	FSHRC collection
Streptomyces coelicolor M145	SCP1-, SCP2-. Contains a mutation of the *sre-I* gene. Wild type	[15]
S. coelicolor L646	*S. coelicolor* M145 contains an integrating plasmid overexpressing wild-type *atrA*, which leads to overproduction of actinorhodin.	[16]
S. coelicolor L645	*S. coelicolor* M145 disrupted *atrA*	[17]
S. coelicolor M511	it is *Δact*II-ORF4	[18]
S.coelicolor M1145	it is *Δact*, *Δred*, *Δcpk*, and *Δcda*	[19]

2.2 Preparation of S. coelicolor spore stocks

S. coelicolor spore stocks were generated according to Jalal [20].

2.3 Streptomyces coelicolor strains integrity

2.3.1 Phenotyping

To investigate the cultural characteristics of the *S. coelicolor* strains, $1x10^5$ spore suspensions from M145, L646, L645, M511, and M1146 strains were spotted on tryptone soya agar (TSA) plates, then kept at 28°C for 6 days, and the colour changes were observed during that time. When the bacterial strains were grown as circular patches (14-16 mm diameter) on TSA media, their phenotypes were compared to each other. Starting on the second day of incubation, the plates were imaged every 24 hours.

2.3.2 Genotyping

2.3.2.1 Preparation of Genomic DNA and purification

Genomic DNA from *S. coelicolor* strains was extracted after 5 days of incubation in a YEME medium containing 10 % sucrose according to [21]. The quantity and quality of the extracted DNA were measured by Nanodrop (Thermo Scientific NanoDrop 2000. SN. 6113) then stored at -20C° until the time of use.

2.3.2.2 Polymerase Chain Reaction (PCR)

To confirm *S. coelicolor* strains' identity, three different sets of primers were designed to target a unique specific region of the certain genomic strain (Table 2). PCR reactions were carried out in a T100™ Thermal Cycler, BIO-RAD (621BR11592, Singapore) using Q5®High-Fidelity 2X Master Mix PCR Kit. The thermal cycling conditions consisted of initial denaturation at 98°C for 5 min followed by 30 cycles of denaturation at 98°C for 10 seconds,

annealing for 30 seconds at various temperatures, and extension at 72°C for 30 seconds. Then the final extension was performed at 72°C for 5 minutes.

Table 2. Represents sequences, and size of primes used in this study and the name of their target genes.

Primer Name	Sequence 5' >>>>>>> 3'	Target gene	Product Size (bp)	Description
atrA –*ACT* gene-for	GGACTCCTCGGACACCAG	*atrA* (SCO4118)	281 bp	differentiateL645 and M1145 strains from the others
atrA –*ACT* gene-rev	GATAGCCGAGGAGGAGACG			
red-prodigosin-for	AGTTCTTCGACCGACGTTTC	*redZ* (SCO5881)	248 bp	differentiateM1145 strain from the others
red-prodigosin-rev	ACGACATGAAAGTGCAGGTG			
*actII*ORF4 - for	GATTCAACTTATTGGGACGTG	*act*II-ORF4 (SCO5085)	746 bp	differentiateM511 strain from the others
*actII*ORF4 - rev	CCGTTGAGAATTTCCATGTG			

2.4 Crude Extraction

To evaluate the antibacterial activities of *S. coelicolor* crude extract, spores of M145 (wild type), L645 (Δ*atrA*, the *act*II-ORF4 regulon), and L646 (has an additional copy of *atrA*) strains were spread on TSA plates separately and incubated at 28°C for 6 days that is when the colour of media turned into blue which is an indicator of actinorhodin production. The agar was collected and mixed with 2.5 volumes of dH_2O before being incubated at room temperature for 1 hour with 200 rpm shaking. The supernatant containing diffusible actinorhodin was collected, and acetic acid was added to lower the pH, causing actinorhodin's colour to shift from blue to red.

The supernatant was extracted with an equal volume of absolute ethyl acetate (Fisher Scientific) and was separated from the aqueous phase by incubating the mixture for 15 minutes at room temperature in a glass separatory funnel. A rotary evaporator (Rotavapor, RE Buchi) was used to evaporate the ethyl acetate, and a parallel evaporator was used to dry the sample (EZ-2 personal evaporator, Genevac) [16]. The crude extract was weighed, then dissolved in 100% (v/v) methanol to a final concentration of 10 mg/mL and kept at 4°C. The crude extract activity was checked as mentioned in sections 2.6.1 and 2.6.2.

2.5 Actinorhodin (ACT) purification

Using a capillary glass tube, aliquots of crude extract dissolved in methanol from strain L646 were spotted on a pencil line about 1 cm from the bottom of a TLC silica gel plate (DC-Alufolien, Kieselgel 60/Kieselgur F254, Merck). Just behind the line where the crude extract loaded, the plate was placed into a TLC tank and submerged in toluene: glacial acetic acid (4:1 [v/v]). The plate was removed once the solvent front reached about 1/3 of the plate, and the solvent front was indicated with a pencil line. The top red band with retention factor (Rf) 0.45 mm was taken out and eluted with ethyl acetate after the plate was dry. The ethyl acetate extract was weighed after drying then methanol was used to dissolve the resultant substance. The Rf value was calculated by dividing the distance travelled by the pigment by the distance travelled by the solvent front. ACT activity was verified as indicated in sections 2.6.1 and 2.6.2 [22-23].

2.6 Antibacterial activity assessment of the crude extract and ACT

Two alternative methods were used to examine the bioactivity of the crude extract and purified ACT.

2.6.1 Disc diffusion assay

Disc diffusion experiment was used to evaluate the antimicrobial activity of dried crude extracts from M145, L646, and L645 strains, as well as purified actinorhodin from the L646 strain [24-25]. Prior to the experiment, 6.0 mm sterile Whatman No. 1 discs were prepared then impregnated with the crude extract or purified actinorhodin at the following concentrations: 100g/ml, 200g/ml, 400g/ml, 600g/ml, and 800g/ml. The discs were placed on overnight Muller-Hinton agar (MHA) (Oxiod) plates that were previously inoculated with 0.1 ml of each of the following standardized test organisms: *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19165, *E. coli* ATCC: 25218, and *Salmonella typhi* ATCC 14028.

Standard antibiotic discs were used to serve as positive controls, which were azithromycin (10 µg/ml) and ciprofloxacin (15 µg/ml). Blank discs impregnated with ethanol solvent were used as a negative control. This experiment was done in triplicate. All the inoculated plates were kept in a refrigerator at 4°C for 1 h to allow the diffusion of the extracts in the media then incubated at 37°C for 24 h to detect and measure the inhibition diameter zone around the discs.

2.6.2 Standard susceptibility test

The Clinical and Laboratory Standards Institute (CLSI) criteria were used to calculate the Minimum Inhibitory Concentrations (MICs) of L646 crude extracts and proposed actinorhodin against the same set of pathogenic bacteria from section 2.6.1 [26]. MICs were read after 18-24 hours of incubation at 37°C. Following incubation, the MIC was defined as the lowest concentration of antibiotic that inhibited all visible growth.

MIC determination with appropriate comparator antibiotics was undertaken to permit comparison of the antibacterial activity of the investigated natural products with existing clinical agents against the tester strains. Positive control (growth control) and solvent control (MHB) were included in each experiment. A susceptibility test was conducted on a minimum of three independent occasions to ensure reproducibility. MIC results were read with high accuracy after incubation, for both groups of crude extract and actinorhodin using an ELISA device (ELX800 ABSORBANCE MICROPLATE READER, BioTek, Germany), and with a Micro-plate (96 wells), at a wavelength of 540 nm.

2.6.3 Data analysis

All data analysis was conducted by Statistical Package for Graphpad prism version 8 in terms of the mean of the growth inhibition zone value obtained from each of the four bacterial pathogens and two standard antibiotics. The data on L646 crude extract and purified actinorhodin extract were analyzed by comparing the mean growth inhibition zone value in disc diffusion test through analysis of variances (one-sample t-test) while in MIC test was analysed by comparing the mean growth inhibition value (Two-way ANOVA). Results with (*P* = *.05*) and (*P-value* summary = ****) were considered as most significant outcome.

3. Results and Discussion

3.1 Streptomyces coelicolor identification approaches

The integrity of *S. coelicolor* strains M145 (the wild type), L645 (Δ*atrA*), M511 (Δ*act*IIOF4), M1164 (Δ*act*, Δ*red*, Δ*cpk*, and Δ*cda*), and L646 (extra copy of *atrA*) were verified using the classical culture method and at the molecular level as follows:

3.1.1 Phenotypic Verification

The phenotype of *S. coelicolor* strains was compared to each other, after 6 days of incubation at 28°C on TSA plates. The representative results of a triplicate set are given in figure 1 as patches with 12-15 mm diameter. The first strain that started actinorhodin production (blue colour) on day 3 was L646 as it contains an extra copy of the regulon *atrA* that regulates *act*II-ORF4, which is in turn synthesis the activator of the cluster-situated act biosynthesis to produce actinorhodin [16]. M145 followed L646 in extracellular production of actinorhodin as it contains a single copy of *atrA* [17]. The actinorhodin blue pigment was not seen in the L645 strain that lacks the *atrA* gene. However, it started prodigiosin production on day 3 [18]. Strains M511 and M1146 did not express any colour changing during the six days of incubation that was expected due to lack of *act*II-ORF4 gene and red cluster in both strains [18-19].

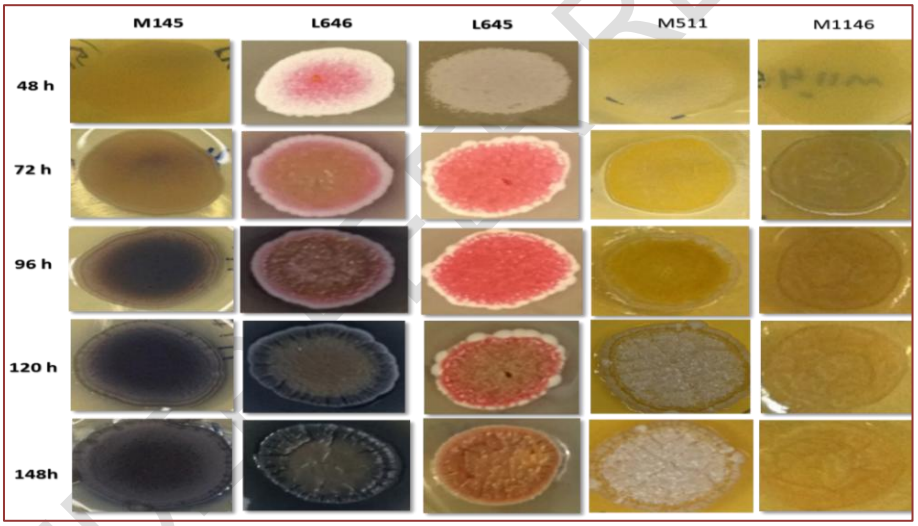

Figure 1. Phenotypic characteristic of *S. coelicolor* strains on TSA. The strains are identified by the top labelling. The hour's period on the left indicates how long the patches had been incubating before being photographed. At least three separate replicates were used to create these images.

3.1.2 Genotypic Confirmation

The molecular method of *S. coelicolor* strains identification started with mycelia collection from the cultured strains then subjected to total DNA extraction. The isolated DNA was analysed using PCR to confirm the identity of the strains. The existence of *atrA* was confirmed using the atrA –*ACT* gene-for and atrA –*ACT* gene-rev primers that target the *atrA* gene in the genome. The expected amplicon of 281 bp was produced for M145, L646, and M511, but not for L645 and M1146 (Figure 2, panel A). The deletion of undecylprodigiosin biosynthetic cluster (RED) was confirmed using red-prodigosin-for and red-prodigosin-rev primers that bind to the chromosomal *redZ* (SCO5881) that activates transcription of *redD*,

the final regulator of the RED biosynthetic cluster. An expected 248 bp PCR amplicon was found for all the strains except M1146 (Figure 2, panel A, lane 5). The presence of the *act*II-ORF4 (SCO5085) gene in the *act* cluster was investigated by targeting it using *act*IIORF4 – for and actIIORF4 – rev primers. A 746 bp of PCR amplicon was detected for M145, L646, and L645 but not for M511 and M1146 (Figure 2, panel C).

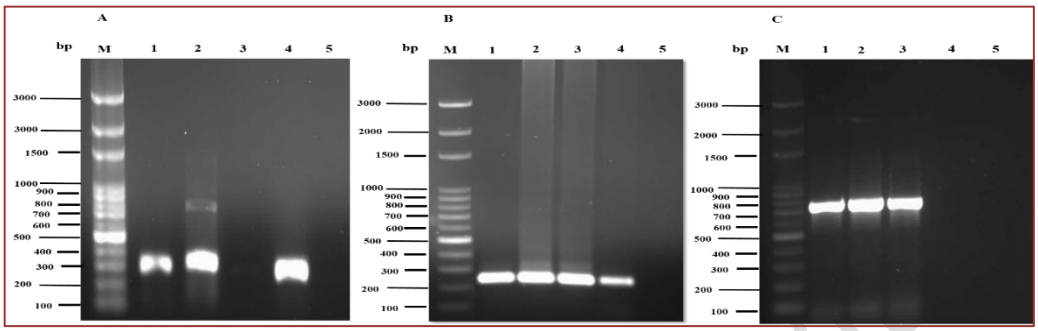

Figure 2. PCR verification of M145, L646, L645, M511, and M1146 strains using (A) atrA –*ACT* gene-for and atrA –*ACT* gene-rev primers that target *atrA* gene (SCO4118), the strains that contain a copy of *atrA*, a 281 bp amplicon was detected, no PCR product was gained for L645 and M1146 strains. (B) red-prodigosin-for and red-prodigosin-rev primers that target *redZ* (SCO5881), a 248 bp amplicon was detected for all strains except M1146, lane 5. (C) actIIORF4 – for and *actII*ORF4 – rev primers that target *actII*-ORF4 gene (SCO5085), a 746 bp PCR product was visualised for M145, L646, and L645, but not for M511 and M1146. In all panels M: contains 100 bp DNA marker (Promega Corporation, USA), lanes 1-5 contains PCR amplicon using a DNA template from M145, L646, L645, M511, M1146 DNA, respectively. A 1.4% agarose gel in 1X TAE was used.

3.2 Chemical molecules Extraction

In order to investigate the ability of *S. coelicolor* to produce biologically active compounds, small molecules were extracted from L646 and L645 strains (as discussed above), then tested against some pathogenic bacteria include *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC19165), *Escherichia coli* (ATCC 25218) *and Salmonella typhi* (ATCC14028).

3.2.1 Chemical Extraction from TS Agar

Chemical extraction contains actinorhodin was isolated from the L646 strain, according to [16] (See section 2.6). L646 strain overproduces actinorhodin as a result of constitutive expression of *act*II-ORF4 from a strong promoter ermE*p, and ribosome-binding site tuf [17]. The extraction proceeded after 6 days of incubation at 28°C when the colour of the TSA plates turned blue, which is an indicator of actinorhodin production (Figure 3, panel A). Same culturing conditions and crude extraction were applied to the *S. coelicolor* L645 strain to be used as a control. Since the L645 strain lacks the *actII*-ORF4 gene, no detectable blue pigment was observed (Figure 3, panel B).

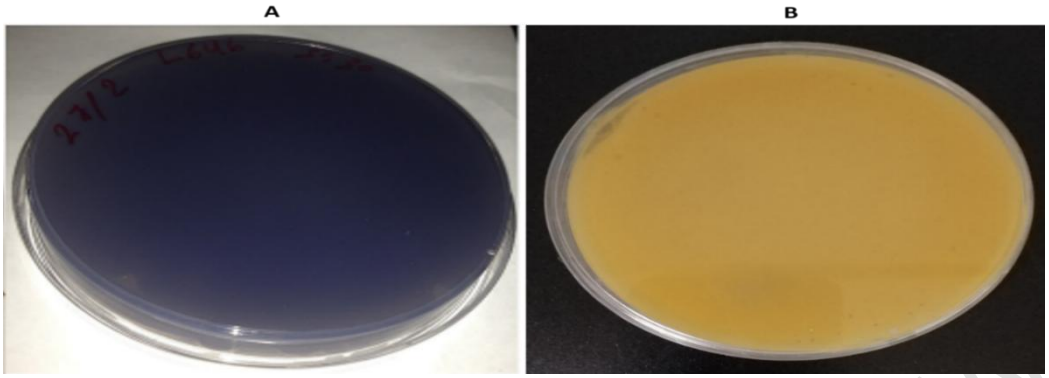

Figure 3. TSA agar plates were inoculated with L646 and L645 strains. Panel A shows the production of blue pigment by L646. Panel B shows L645 culture with no change in the colour as an indicator of no actinorhodin production. The plates were incubated for 6 days at 28°C.

The extracted chemicals were concentrated, dried, weighed, and then re-suspended in methanol at a known concentration. A pilot study was set to evaluate the validity of the extracted chemicals before proceeding to any further investigation. Different quantities of the crude extract were prepared, dried and suspended with methanol then spotted at certain concentrations onto 6 mm diameter filter papers, which in turn they placed on the surface of MHA agar plates that have been cultured with *Staphylococcus aureus* (ATCC 25923) and incubated at 37°C for 24 h. The results confirmed that the chemical extract that contains actinorhodin from L646 has the ability to inhibit *Staphylococcus aureus* by generating a clear zone ranging between (14-25mm) around the discs at 100µg/ ml (Figure 4, panel A). This activity was absent when a crude extract from the L645 strain was used in the experiment (Figure 4, panel B) since L645 does not possess the *atrA* gene that activates actinorhodin production.

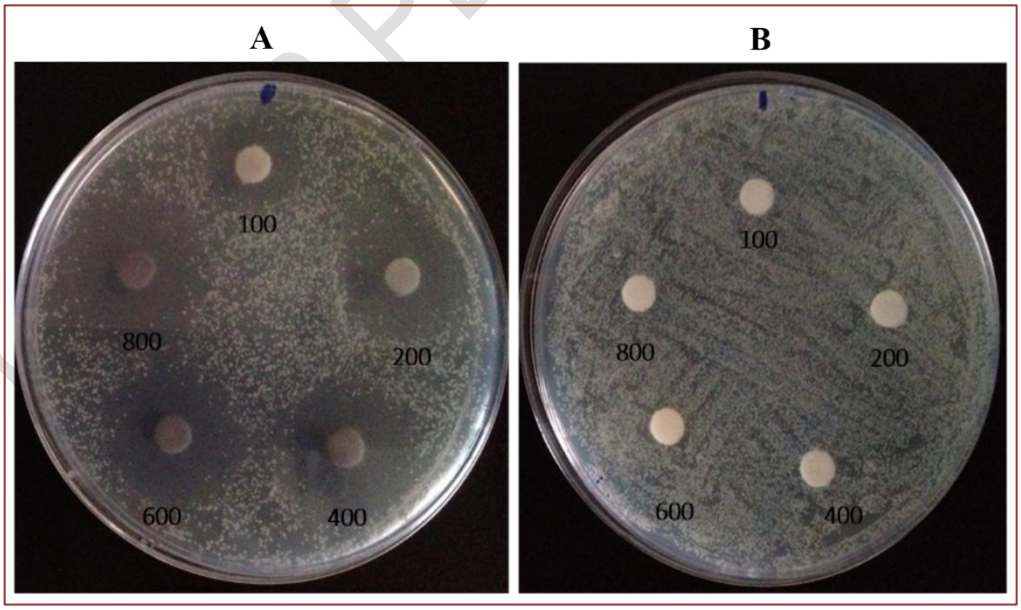

Figure 4. Shows the impact of crude extract from L646 and L645 against *Staphylococcus aureus* (ATCC 25923). Panel (A) represents the effect of the crude

compound from L646 that shows a clear zone around the tested discs. Panel (B) represents a crude compound from *S. coelicolor* L645 with no effect. 100, 200, 400, 600, 800 refer to the used concentrations as µg/ml or what) of the crude extract on MHA medium. All the pales were incubated overnight at 37°C.

3.2.2 Thin-layer chromatography (TLC) for actinorhodin (ACT) purification

As an insight was obtained from the previous experiment (Section 3.2.1), which was the inhibition ability of actinorhodin containing crude, we attended to prepare quantities of the active compound that we believe is actinorhodin based on a previous study [27]. Thin-layer chromatography (TLC) plate with toluene: acetic acid [v / v (4: 1)] as a solvent was used for this purpose (see Section 2.5). The proposed actinorhodin was collected at a retention factor (R_f) of 0.45 mm [28] (Figure 5). The proposed actinorhodin was concentrated, air-dried, weighed, and then re-suspended in methanol at a known concentration.

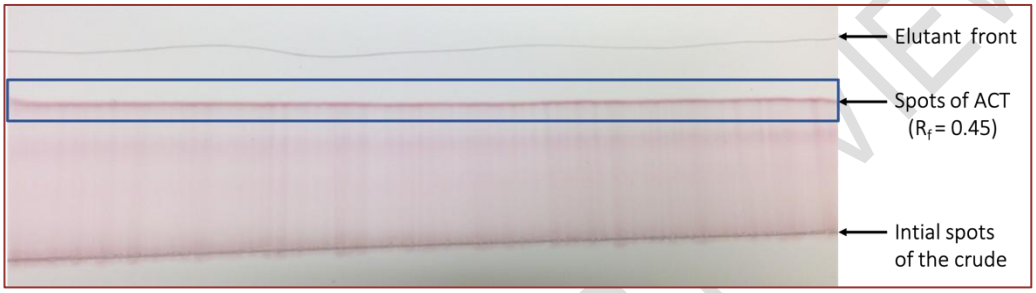

Figure 5. Represents the position of the actinorhodin band that has been removed from the TLC plate. A blue box indicates the actinorhodin band that has been removed from the TLC plate. Toluene: acetic acid [v/v (4:1)] was used as a solvent. The R_f value is (0.45) mm.

3.3 Antibacterial activity of the crude extract and proposed actinorhodin

The crude extract and suggested actinorhodin (ACT) from the L646 strain were used against the mentioned set of pathogenic gram-positive and gram-negative bacteria at different concentrations by two methods, which were disc diffusion assay and minimum inhibitory concentration assay.

3.3.1 Disc Diffusion

Disc diffusion assay of crude extract and proposed actinorhodin showed significant differences against some important pathogens such as *Staphylococcus aureus* ATCC 25923*, Streptococcus pyogenes* ATCC: 19165, *E. coli* ATCC: 25218, and *Salmonella typhi* ATCC: 14028, on MHA medium. The inhibition zone size around the discs was used as an indication of the efficacy of the tested extracts on bacterial growth. Ciprofloxacin (CIP) and azithromycin (AZM) were used as positive controls at 10 µg/ ml and 15 µg/ ml, respectively.

It was noted that the crude extract has an effect as an inhibitor against *Streptococcus pyogenes* and *Staphylococcus aureus* in standard conditions with diameter zone inhibition of (11-23 mm) and (9-20 mm), respectively, while fewer inhibition activities were shown against *E. coli* with diameter zone inhibition of (8-16 mm) and no effect was detected against *S. typhi* (figure 6 panel A). However, actinorhodin showed a reasonable wider effect against them with diameter zone inhibition of (10-27 mm) against *S. pyogenes* and (10-24 mm) against *S. aureus*; while less effect against *E. coli* with diameter zone inhibition of (8-17 mm) was detected and no effect was determined against *S. typhi* (figure 6, panel B). In all cases, the proposed actinorhodin was shown considerately more activity against the tested pathogens compared to the crude extract.

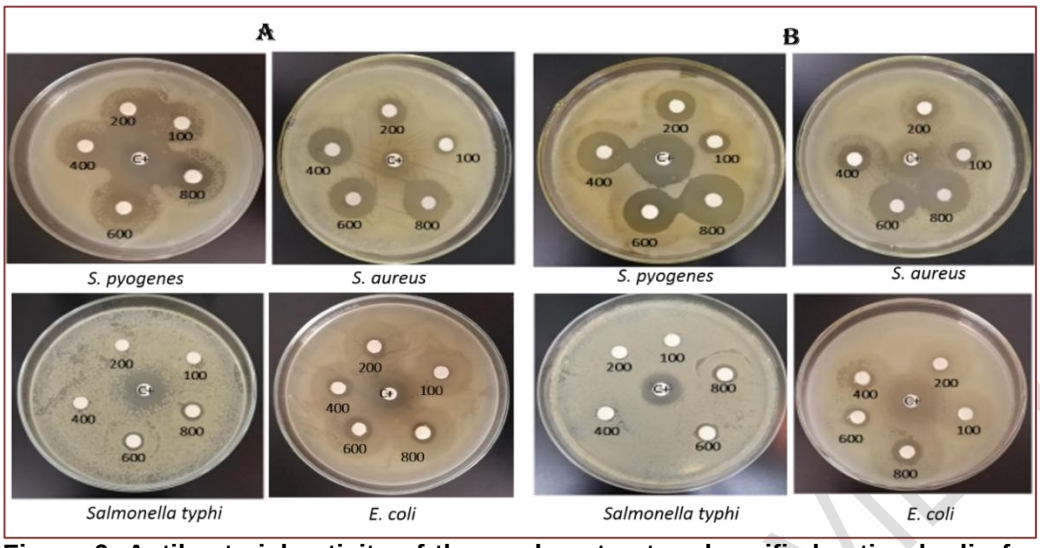

Figure 6. Antibacterial activity of the crude extract and purified actinorhodin from *S. coelicolor* L646 by disc diffusion test. Panel (A) shows the use of crude extract against four pathogenic bacteria at different concentrations (100, 200, 400, 600, 800 µg/ ml). In parallel, actinorhodin was used in panel (B) in the same manner and conditions as in panel A. All the tests were performed on MHA medium and the results were taken after 24 h of incubation at 37°C. C^+ refers to a positive control (ciprofloxacin and Azithromycin).

3.3.2 Minimum inhibitory concentrations MICs

MICs of the crude extract and purified actinorhodin were determined for several clinically important Gram-positive and Gram-negative bacteria which included *Staphylococcus aureus*, *Streptococcus pyogenes*, *E. coli,* and *Salmonella typhi* using the standard CLSI broth microdilution method. The crude extract exhibited potent antibacterial activity against *Staphylococcus aureus* and *Streptococcus pyogenes*, giving MIC values of 16 µg/ml. However, it showed less antibacterial activity against *Salmonella typhi* and *E. coli* with MICs values of (64 and 128 µg/ml), respectively (Figure 7). The results fall within the comparable range for systemically administrated antibacterial drugs in current clinical use such as azithromycin and ciprofloxacin, which were given a MIC value of 4 µg/ml against *S. aureus* and *E. coli*, respectively.

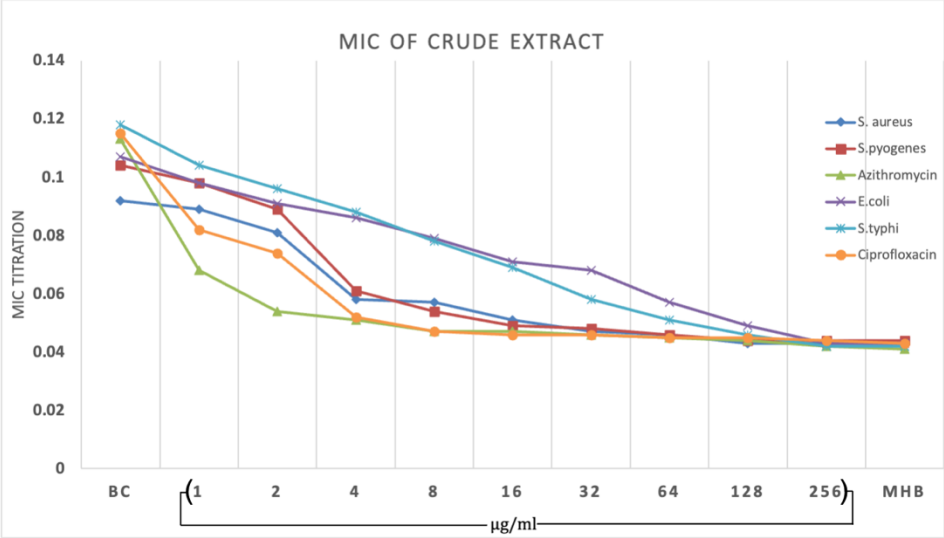

Figure 7. A statistical chart representing minimum inhibitory concentration (MIC) of the crude extract from *S. coelicolor* L646 against *S. aureus*, *S. pyogenes*, *S. typhi* and *E. coli* after 18-24 h of incubation at 37°C in MH broth. BC stands for the antibiotic-free bacterial culture that is set as positive control and MHB stands for bacterial free Mueller Hinton Broth that has been established as a negative control.

On the other hand, MICs were determined for the purified actinorhodin against the same group of bacteria following the same conditions and procedures. Actinorhodin expressed notable antibacterial activity against *S. aureus*, *S. pyogenes* and *S. typhi* with MICs of (8, 16 and 32 µg/ml), respectively. However, it gave a MIC value of 128 µg/ml against *E. coli* as shown in figure (8). The above results fall within the comparable range for systemically administrated antibacterial drugs in current clinical use such as chloramphenicol and ciprofloxacin which display MIC values of 4 µg/ml against susceptible *S. aureus* strains.

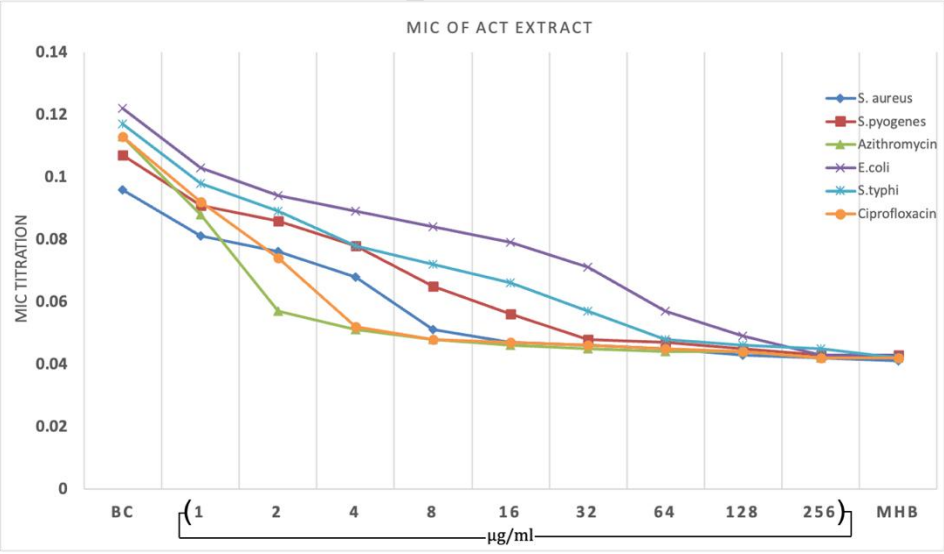

Figure 8. A statistical chart representing minimum inhibitory concentration (MIC) of the purified actinorhodin isolated from *Streptomyces coelicolor* L646 against *S. aureus*, *S. pyogenes*, *S. typhi* and *E. coli* after 18-24 h. BC represents antibiotic-free

bacterial culture, which was used as positive control and MHB represents bacterial free Mueller Hinton Broth, which was used as a negative control.

Although secondary metabolite synthesis is not required for *Streptomyces* to survive, these compounds provide an evolutionary advantage over other occupiers since they may be employed as a chemical agent to positively influence other microorganisms. Since *Streptomyces* genus has been discovered to be a reliable source of bioactive compounds with human health applications, much effort is being put into isolating novel pharmacologically active compounds from this genus [29]. As a result, we focused our work on whether *Streptomyces coelicolor* can produce any antibacterial active molecules through their biosynthetic pathways.

Our data indicated that the crude extract from *Streptomyces coelicolor* possesses significant antibacterial activity against representative gram-positive bacteria, with MIC values of 16 µg/ml. However, it lacked useful antibacterial efficacy against gram-negative pathogens, with MICs of >128 µg/ml against the tested gram-negative bacteria. It was expected from previous pilot studies by our group that actinorhodin is the active compound that expresses this antibacterial activity. So, we attempted to examine its biological activity after isolating it from the crude extract of *S. coelicolor* strain L646 that overexpresses *atrA*, which results in an increase in actinorhodin production. A prominent, well-separated purple band with R_f 0.45 was obtained when the crude chromatographed on TLC (Figure 5). This proposed actinorhodin showed good efficacy against *S. aureus* with a MIC of 8 µg/ml based on the MIC value for the clinically useful antibiotic azithromycin against the same bacterial genus with a MIC of 4 µg/ml [27]. Against *S. pyogenes* and *E. coli*, the actinorhodin had the same effect as the crude extract. The ACT and the crude extract lacked efficacy against *S. typhi* with MICs between 32-64 µg/ml, respectively and *E. coli* with MICs of >128 µg/ml.
Compared to the Gram-positive pathogens, both extracts showed less effectiveness against the representative of Gram-negative pathogens that could be due to the architecture differences in their cell wall structure, particularly the peptidoglycan content. Furthermore, gram-negative bacteria have a lipopolysaccharide membrane on the exterior of their cell wall that makes it impenetrable [30-31]. According to [32], actinorhodin's poor efficacy against Gram-negative bacteria is due to its restricted ability to traverse the outer membrane and reach its target site, or it could be due to the action of the existence of substrate specificity range efflux pumps such as AcrAB-TolC [27-33].
Our findings are in line with those of several previous studies, such as one by [28], in which they partially purified 1 mg of crude from *Streptomyces ruber* EKH2 using a silica gel plate by TLC technique. Different fractions were obtained with various (R_f) values, however, the one with R_f of 0.45 was more effective against a broad range of medically important bacteria. Another study by [34-35] supported our finding by confirming that the purified actinorhodin from *Streptomyces coeruleorubidus* BTSS-301 by TLC method showed high effectiveness against gram-negative tested pathogens including *Staphylococcus aureus* and *E. coli* using minimum inhibitor concentration (MIC) of the partially purified compound.
The disc diffusion experiment results of the crude extract and suggested actinorhodin confirms the MIC findings, which is backed by [36], which found that the extracted and purified material by TLC was effective against the organisms tested in both the disk diffusion and MIC assays equally. The antistaphylococcal activity of actinorhodin demonstrated here is substantially higher than that previously reported for actinorhodin, the original work reported antibacterial activity against *Staphylococcus* with a MIC of 25-30 µg/ml using the agar diffusion method [37]. This obvious difference might be explained by a number of factors. The first factor can be that the MIC value was calculated in the original study using the agar plug diffusion technique. Briefly, the actinorhodin producer bacteria were grown on a complete medium (CM) for 48 h, and then 6 mm diameter colony centred plugs of CM agar were cut and moved into a Petri dish filled with nutrient agar containing *S. aureus*. In contrast, we used broth culture to estimate the MICs values using ELISA. So, the

considerable disparity in the MICs achieved is most likely due to the choice of culture medium. The second factor can be returned to the differences in actinorhodin analogues that can express various levels of bioactivity against *S. aureus*.

This study demonstrates the value of revisiting an old mine to re-evaluate an excitant natural product (e.g., actinorhodin) in the quest for novel antibacterial reagents. The finding of this study supports such action through proposing actinorhodin as an active future antibiotic against staphylococcal infection, which we believe acts on the bacterial membrane differently from other membrane-perturbing agents used in clinical practice [38].

4. Conclusion

In conclusion, this work has examined the antibacterial activity of *S. coelicolor* secondary metabolites. The remarkable result that we obtained from studying small molecules was the ability of proposed actinorhodin from the L646 strain (figure 8), in which an extra *atrA* gene has been added, to inhibit *S. aureus* and *S. pyogenes* growth with a MIC value of 8 µg/ml. However as yet, the small molecule(s) exact structure is unknown. The next step would be to fractionate a crude extract from the L646 strain and screen the fractions against some human pathogenic bacteria to identify the fraction containing the inhibition activity. Analytical LC-MS may be used to produce a high-quality molecular weight from the most active fraction, which could then be utilized to create a formula. Finally, NMR might be carried out to identify the small molecule's physical and chemical characteristics.

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COMPETING INTERESTS

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the personal efforts of the authors.

AUTHORS' CONTRIBUTIONS

Author A' and B' designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. 'Author A' managed the analyses of the study. 'Author B' managed the literature searches… All authors read and approved the final manuscript.

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