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# Elicitation of Cryptic Secondary Metabolites and Antibacterial Activities from Mangrove and Cave Soil Actinomycetes

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## ABSTRACT

Actinomycetes' secondary metabolites have received considerable attention due to their many beneficial biological activities. However, many biosynthetic gene clusters in actinomycetes remain silent as they are not transcribed under standard laboratory conditions. Therefore, this study aims to introduce antibiotic elicitors to activate cryptic secondary metabolites in soil actinomycetes and screen them for antibacterial potential. A total of 20 cave and 10 mangrove soil actinomycete isolates were exposed to streptomycin or erythromycin at subinhibitory concentration (0.5–1048 µg/mL) in minimal media. The ethyl acetate extracts were subjected to high-performance liquid chromatography (HPLC) profiling to observe the effect of elicitors towards secondary metabolite production. As a result, 61.7% of the isolates showed a positive impact (appearance of 'new'/increase in metabolite production) when elicitors were supplemented. These changes were more pronounced in erythromycin-induced media (63.3%) than in streptomycin (60.0%). Two isolates (CS3PT50 and CS3PT53) exhibited significant changes in the profile, with additional peaks detected at 210 and 245 nm, which may indicate the production of new metabolites. More antibacterial activities were observed from stimulated (26.7%) as opposed to non-stimulated isolates (10.0%), including 6 new activities, 1 improved, and 1 decrease in inhibitory. Furthermore, isolate

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CS3PT53 (0.5 mg/disc) displayed broad-spectrum activities, inhibiting *S. aureus* ATCC 25923 and *S.* Typhimurium ATCC 14028. The hit actinomycete isolates belonged to the genus *Streptomyces* (55.6%), *Norcardia* (22.2%)*, Norcardiopsis,* and *Saccharomonospora* (11.1%). Overall, this study demonstrated that incorporating antibiotic elicitor at subinhibitory concentration could effectively trigger the production of cryptic secondary metabolites with antibacterial properties in soil actinomycetes.

*Keywords:* Antibacterial activities, cryptic secondary metabolites, elicitors, soil actinomycetes

## INTRODUCTION

Actinomycetes are a diverse group of filamentous, spore-forming Gram-positive bacteria broadly distributed in nature. They are factories of many important secondary metabolites, biologically useful enzymes, and lead molecules, which have found application, particularly in the medical, pharmaceutical, and veterinary sectors (Salwan & Sharma, 2020). More than half of the naturally derived antibiotics currently available in the market originated from actinomycetes (Barka et al., 2016; Ochi, 2017). However, the chances of discovering new bioactive molecules from known actinomycetes have gradually reduced due to the saturation effect (Lee et al., 2014). Exploring unique natural settings such as mangrove and cave soils has resulted in the discovery of more than 220 non-*Streptomyces* or rare actinomycetes, with over a quarter producing 2500 bioactive metabolites (Ezeobiora et al., 2022; Jiang et al., 2016). Despite the potential, rediscovering known compounds or metabolites from actinomycetes remains a major concern (Tomm et al., 2019).

Although actinomycetes produce a wide array of natural metabolites with complex scaffolds and diverse therapeutic effects, a large portion of these molecules encoded in the actinomycete genomes remain unexplored (Covington et al., 2018). It is presumably because these genes are seldom expressed or expressed poorly, with the majority remaining phenotypically silent under conventional screening conditions (Abdelmohsen et al., 2015; Begani et al., 2018). A single *Streptomyces* strain is estimated to produce around 4 compounds but encodes 20–50 secondary metabolite pathways (Belknap et al., 2020; Caboche, 2014; Yagüe et al., 2022). Conversely, other actinomycete families, including *Corynebacteriales*, *Micromonosporaceae*, *Streptosporanqineae*, as well as *Pseudonocardiales*, possess approximately 8.4, 13.3, 15.0, and 19.8 secondary metabolite biosynthetic gene clusters (BGCs) per genome and only a fraction of the compounds have been isolated using standard fermentation conditions (Doroghazi et al., 2014). Therefore, exploiting different practical means to induce these silent or cryptic BGCs is of major interest as it may lead to identifying novel scaffolds with targeted biological activities.

At present, various methods have been utilised to activate novel natural products from cryptic BGCs, including the one strain many compounds (OSMAC), chromatin remodelling,

high-throughput elicitor screening (HiTES), genome mining, physical/environmental stress (exposure to UV, changing temperature, and pH shock), co-cultivation with bacteria or fungi and incorporating chemical elicitors (rare earth elements, epigenetic modifiers, organic solvents, and nanoparticles) (Balagurunathan et al., 2020; Begani et al., 2018; Zong et al., 2022). The last approach is among the actively pursued methods as it involves a defined, single chemical agent that triggers a biological response and can easily be optimised to promote existing or new metabolites in actinomycetes (Akhter et al., 2018; Imai et al., 2015; Shentu et al., 2016). According to Narayani and Srivastava (2017), elicitors with the appropriate concentration will induce stress, thus resulting in the activation or inactivation of defence-related genes, transient phosphorylation or dephosphorylation of proteins, and upregulate or even downregulate important enzymes needed for the expression of specific secondary metabolite BGCs.

Growing evidence has revealed that antibiotics at subinhibitory concentrations can potentiate antibiotic production in different *Streptomyces* species (El-Hawary et al., 2023). For example, Imai et al. (2015) reported that a number of antibacterial compounds, including the well-known antibiotic actinorhodin, were induced in the methanolic extract of *S. lividans* 1326 when lincomycin was incorporated at subinhibitory concentration (1/12 or 1/3 of its MIC). The production of the corresponding antibiotic was also found to be dose-dependent, with 5 µg/mL being the ideal concentration. In a separate study by Zhang et al. (2015), cryptic gene clusters in *S. somaliensis* ZH66 were triggered and activated by an anticancer drug lead, fredericamycin A. It was accomplished by adding 300 µg/mL of rifampicin to the fermentation medium, and after 7 days of incubation, a considerable yield (679.5 ± 15.8 mg/L) of the bioactive compound was obtained. The high-dose toxicity, low-dose stimulatory effect is known as hormesis, where at optimum sub-lethal doses, antibiotics or other bioactive molecules can have pleiotropic effects on multiple unrelated bacterial gene expression (Davies et al., 2006; Okada & Seyedsayamdost, 2017). Here, we applied subinhibitory antibiotic concentration (streptomycin and erythromycin) to 30 soil actinomycete isolates. The secondary metabolite responses, as well as their antibacterial potential against four pathogens were assessed. The results were compared between stimulated and non-stimulated HPLC profiles to highlight the effect of antibiotic elicitors on the activation of cryptic secondary metabolites and their antibacterial potential.

## MATERIALS AND METHODS

### Bacterial Strains

The mangrove soil actinomycetes used in this study were previously obtained from Ariffin et al. (2017), while the cave soil actinomycetes were isolated from two cave soil samples (Gua Kelawar and Gua Wang Buluh) collected from Pulau Tuba Kedah, Malaysia in 2019.

For antibacterial screening, the test bacteria *Escherichia coli* ATCC 25922, *Salmonella enterica subsp. enterica* ser. Typhimurium ATCC 14028, *Staphylococcus aureus* ATCC 25923, and *Bacillus subtilis* ATCC 6633 were obtained from the American Type Culture Collection.

**Revival of Actinomycete Isolates**

Two selective growth media, tryptone yeast extract (ISP 1) and yeast malt extract medium (ISP 2), were prepared. For mangrove actinomycetes, 3% (final concentration) of sodium chloride (NaCl) was supplemented to replace seawater. All ingredients were dissolved in distilled water and adjusted to pH 7.0–7.2 prior to sterilisation. The actinomycetes glycerol stocks were then thawed, and 100 µL of bacterial culture was pipetted into a universal bottle containing 8 mL of ISP 1 (1.5 g yeast extract and 2.5 g tryptone) and ISP 2 (5.0 g malt extract, 2.0 g yeast extract, and 2.0 g glucose). Broths containing actinomycete isolates were incubated at 30ºC for 14 days (170 rpm) and observed for growth. Pure actinomycete isolates were streaked and maintained on ISP 2 agar.

**Determination of Elicitors' Minimum Inhibitory Concentration (MIC)**

The elicitors' MIC values for each actinomycete isolate were pre-determined using the agar well diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2021) guidelines with slight modification. The isolates were cultured in ISP 2 broth and incubated at 30ºC for 14 days (170 rpm). The bacterial suspension was adjusted to 0.5 McFarland standard before swabbing onto the minimal media (glucose 0.25 g, yeast extract 0.25 g, $K_2HPO_4$ 0.50 g, NaCl 0.25 g, $MgSO_4.7H_2O$ 0.25 g, agar 15 g, pH 7.5–8.0). A 7 mm hole was punched aseptically with a cork borer, and 50 µL of the antibiotic elicitor, streptomycin, was introduced into the well with concentrations ranging from 0.25–2048 µg/mL. The agar plates were then incubated at 30ºC for 14 days before the inhibition zones were measured in millimetres. 5 µg/mL of ciprofloxacin was used as the positive control, while 50 µL of sterile distilled water was the negative control. The concentration that inhibited actinomycetes growth completely was taken as the MIC value. All tests were carried out in triplicates, and similar procedures were applied to the erythromycin.

**Fermentation of Actinomycete Isolates with and Without Elicitors**

The revived actinomycete isolates were cultured into minimal media, and antibiotic elicitors (streptomycin and erythromycin) were added separately at a subinhibitory concentration (½ MIC value determined previously) to trigger cryptic secondary metabolites. For the seeding material, a pure colony of each actinomycete isolate was spread evenly onto ISP 2 agar using a sterile cotton swab and incubated at 30°C for 14 days. After incubation, 2 holes with a diameter of 7 mm were punched aseptically with a cork borer. The agar plugs

were then seeded into a 500 mL Erlenmeyer flask containing 250 mL of fermentation media and incubated at 30°C for 14 days (170 rpm). The above methods were repeated without the addition of elicitors. This served as a control for comparison purposes.

## Extraction of Secondary Metabolites

After fermentation, each culture was filtered (Whatman No.1 filter paper), and the resulting cell-free filtrate was collected. Liquid-liquid extraction was carried out by mixing an equal volume of ethyl acetate 1:1 (v/v) with the filtrate and shaking vigorously for 2 hours. The organic layer was collected and separated from the aqueous layer using a separating funnel. This process was repeated three times, and the organic layer was concentrated using a rotary evaporator. All crude extracts were transferred into pre-weighed vials, air-dried, and weighed.

## HPLC Profiling of Crude Extracts

Samples were prepared freshly by dissolving the crude extract in 70% acetonitrile (HPLC grade) to make a 1 mg/mL concentration. Similar procedures were also employed for both antibiotic elicitors. Each solution (1 mL) was filtered through a 0.22 µm PTFE filter before injecting into the HPLC system. An injection volume of 10 µL was used. HPLC analysis was performed using Dionex Ultimate 3000 chromatographic system (Thermo Scientific, Bremen, Germany) with rapid separation diode array detector and separated on a Phenomenex Luna C18 column (4.6 mm × 250 mm, 5 µm) with acetonitrile/water as the mobile phase, applying a gradient of 10-100% acetonitrile over 42 mins, using a flow rate of 0.8 mL/min (Sadiran, 2011). The column temperature was set at 30°C, and the secondary metabolite profiles were monitored at multiple wavelengths (210, 245, 270 and 366 nm). Differences in chemical profiles were then compared between the stimulated and non-stimulated actinomycete isolates based on the UV absorption peaks and retention time ($t_R$).

## Antibacterial Activities of Crude Extracts

The antibacterial activities of the crude extracts were evaluated using the standard disc diffusion method following the CLSI (2021) guidelines. The test pathogens were cultured in Mueller Hinton broth and incubated for 16–24 hours at 37°C. The cultures were subsequently adjusted to an $OD_{625nm}$ of 0.08-0.10 (equivalent to 0.5 McFarland standard) before swabbing onto Mueller Hinton agar (MHA). For this assay, ciprofloxacin (5 µg/disc) was used as the positive control, while 10% dimethyl sulphoxide (DMSO) was used as the negative control. A cell-free minimal media incorporated with streptomycin or erythromycin (256 µg/disc respectively) was also included as one of the controls to ensure that the activity observed is due to the crude extracts alone and not the elicitors used. All crude extracts were dissolved in 10% DMSO to produce a working concentration of 10

mg/mL. Sterile blank discs (6 mm) were impregnated with 50 µL of extract, equal to a final concentration of 0.5 mg/disc. The saturated discs were then placed on the surface of the MHA plate containing the test bacteria and incubated at 37°C for 24 hours. The assay was conducted in triplicates. The zone of inhibition was measured to the nearest millimetre (mm).

### Molecular Identification of the Hit Actinobacteria Isolates

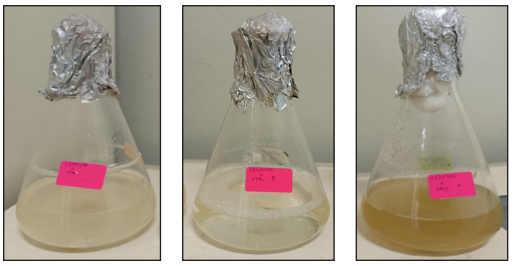
Actinomycete isolates that displayed antibacterial activities were sent to the third party (Apical Scientific Sdn. Bhd., Malaysia) for DNA extraction, 16S rRNA gene amplification, PCR purification and sequencing. The DNA was extracted using the Presto™ Mini gDNA Bacteria Kit (Geneaid, GBB100). The 16S rRNA (1.5 kb) was amplified using universal primers (27F and 1492R). The identity of the isolates was determined using the Basic Local Alignment Search Tools (BLAST) and compared to the known species in the GenBank database using the National Centre for Biotechnology Information software (www.ncbi.nlm.nih.gov/).

## RESULTS AND DISCUSSION

### Effect of Antibiotic Elicitors on Secondary Metabolite Production

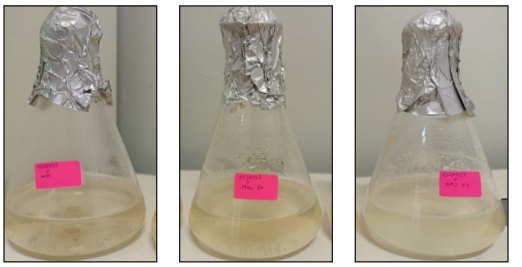
This work exposed 30 morphologically distinct actinomycete isolates recovered from different mangrove and cave soils around Malaysia to antibiotic elicitors, streptomycin and erythromycin separately. In order to determine the subinhibitory concentration needed during fermentation, the elicitors MIC towards each isolate was pre-determined. This was necessary to ensure that the concentration used was not lethal but enough to create environmental stress towards the selected actinomycete isolates. Based on the results gathered, most of the isolates tolerated streptomycin (MIC value ranging from 0.5–256 µg/mL) compared to erythromycin (MIC value ranging from 8–2048 µg/mL). Isolate CS3PT5 and PGS123 were the most resistant to erythromycin and streptomycin, respectively. After 14 days of fermentation, phenotypic differences such as colony size, colony pigmentation, and turbidity of the media were observed. In general, isolates exposed to elicitors showed delayed or slow growth initially, with smaller colonies but higher biomass, which resulted in a turbid fermentation media. The size difference is likely due to the actinomycetes' response to nutrient limitation and environmental stress (Maier & Pepper, 2015). Furthermore, the turbidity may be attributed to the inducing growth effect during the late growth phase when subinhibitory concentration was applied (Imai et al., 2015). Phenotypic changes were observed for both treated media compared to the control broth. As shown in Figure 1, media supplemented with erythromycin showed brownish pigment, while the streptomycin-treated media exhibited

white-pinkish pigmentation. Both broths supplemented with elicitors were also more turbid, suggesting environmental adaptation. A similar outcome was also observed for isolate CS3PT53 (Figure 2), where the media containing streptomycin showed light orange pigmentation. In contrast, the others were off-white (media with erythromycin) and leaning towards transparent broth (control media). Both media containing elicitors were likewise more turbid than the control media. Differences in colony sizes were also clearly observed. Such an outcome was also reported by Wang et al. (2017), where spectinomycin altered the morphology and pigmentation of *S. coelicolor* M145. Without this antibiotic, *S. coelicolor* M145 grows protruding hyphae and forms large pellets during fermentation. However, when spectinomycin was incorporated, pallet diameters were smaller, while the hyphae formation was barely visible.



(a) (b) (c)

*Figure 1.* Isolate CS3PT50 after 14 days of fermentation: (a) control media (without elicitor); (b) media supplemented with streptomycin (8 µg/mL); and (c) media supplemented with erythromycin (4 µg/mL)



(a) (b) (c)

*Figure 2.* Isolate CS3PT53 after 14 days of fermentation: (a) control media (without elicitor); (b) media supplemented with streptomycin (32 µg/mL); and (c) media supplemented with erythromycin (64 µg/mL)

In order to assess the effect of elicitors towards secondary metabolite production, all crude extracts were subjected to HPLC analysis, and the chromatograms were compared between stimulated and non-stimulated fermentation media. Due to the absence of chromophores in both elicitors, their chromatograms were not included for comparison. Observations were made in terms of the presence of new or additional peaks, enhanced or reduced peak intensities, loss of peaks, and no change. The different profile patterns gathered for each actinomycete isolate are summarised in Table 1. Based on the results tabulated, changes in the secondary metabolite profiles were observed for all the isolates (100%, 30/30) when elicitors were added to the fermentation media. Due to the complex secondary metabolism in actinomycetes, most of the isolates (66.7%, 20/30) exhibited a combination of patterns instead of a single change when streptomycin was incorporated, while for erythromycin, mixed patterns were seen in 43.3% of the isolates (13/30). Moreover, streptomycin elicited 'new' secondary metabolites in 14 isolates (46.7%), whereas such changes were only observed in 10 isolates (33.3%) when erythromycin was

supplemented. It is also important to note that 17 isolates (56.7%) lose their original peaks compared to the control media profiles, suggesting that the elicitors likewise suppress the production of certain metabolites. The variation in patterns corroborated previous reports where the usage of ribosome-targeting antibiotics tends to produce different responses across the actinobacterial genera (Covington et al., 2018; Shentu et al., 2016). In summary, 40% (24/60) of the isolates activated 'new' secondary metabolites and 56.7% (34/60) enhanced the production of the original metabolites. The proportion of positive impacts (appearance of 'new' metabolites/ increase in metabolite production) is higher than the negative impacts (loss of original metabolites/ decrease in metabolite production), which are 61.7% (37/60) and 43.3% (26/60) respectively.

Table 1
*The different pattern(s) in secondary metabolite profiles of the stimulated and non-stimulated media*

| Peak pattern(s) | Streptomycin | Erythromycin | Total |
|---|---|---|---|
| **New** | 2/30 | 1/30 | 3/60 |
| **Enhance** | 4/30 | 9/30 | 13/60 |
| **New/ enhance** | 12/30 | 9/30 | 21/60 |
| **Reduce** | 3/30 | 6/30 | 9/60 |
| **Loss** | 2/30 | 3/30 | 5/60 |
| **Loss/ reduce** | 8/30 | 4/30 | 12/60 |
| **Change** | 30/30 | 30/30 | 60/60 |

*Notes*. The numbers represent the number of isolates

Among the 30 isolates exposed to the elicitors, two isolates (CS3PT50, CS3PT53) demonstrated a distinct inducing effect in the HPLC profiles where additional peaks were detected at 210 and 245 nm. The HPLC profiles of both isolates are shown in Figures 3 and 4, respectively. The metabolic profiles of isolate CS3PT50 at 245 nm (Figure 3) showed that more than 3 new peaks could be detected between retention time ($t_R$) 16.0 to 27.0 min when streptomycin was incorporated. Apart from 2 enhanced peaks (minute 27.0 and 36.0), a peak loss at 11.5 min can also be observed from the chromatograms. As for isolate CS3PT53, a new peak appeared at minute 13.5, while 2 peaks were enhanced (22.5 min and 35.5 min) in the presence of streptomycin. However, two important peaks (minute 8.0 and 9.0) disappeared when both antibiotic elicitors were added. The differences in the chemical profiles indicated that new cryptic secondary metabolites may be triggered when environmental stimuli were introduced. It is also worth noting that these 'new' metabolites may likewise be derivative(s) of the elicitors and could result from biotransformation. In addition, none of the antibiotic concentrations in this work was optimised during fermentation and, thus, should be carried out in the near future to increase the metabolite production of new peaks.

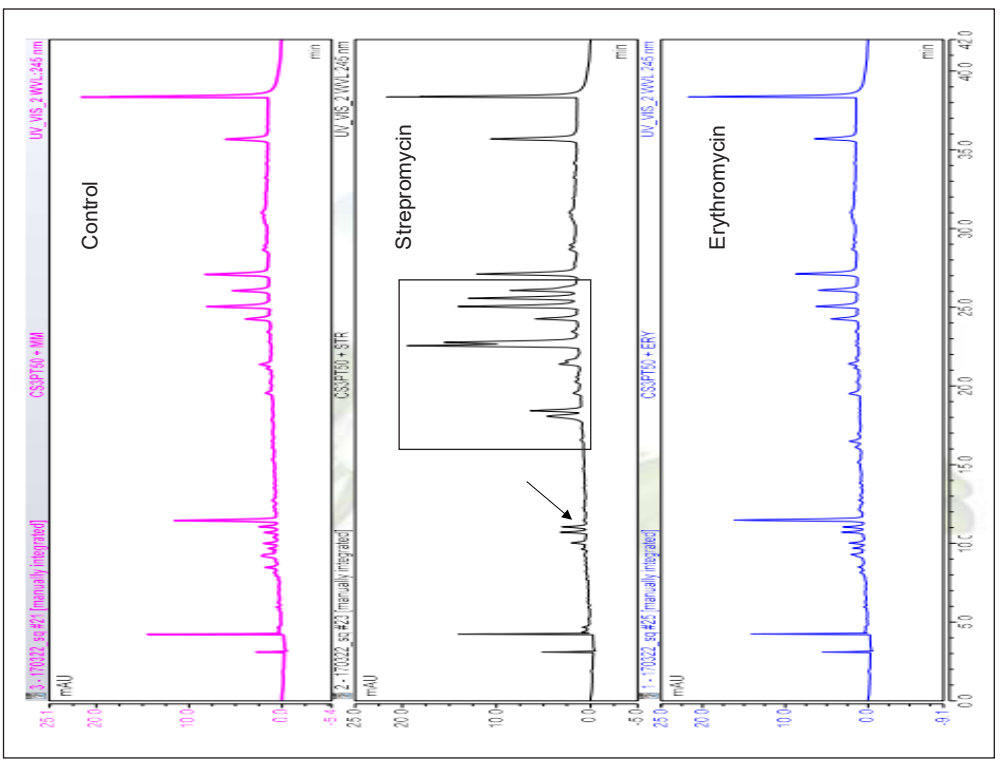



*Figure 3.* HPLC profiles of isolate CS3PT50 extracts at 245 nm

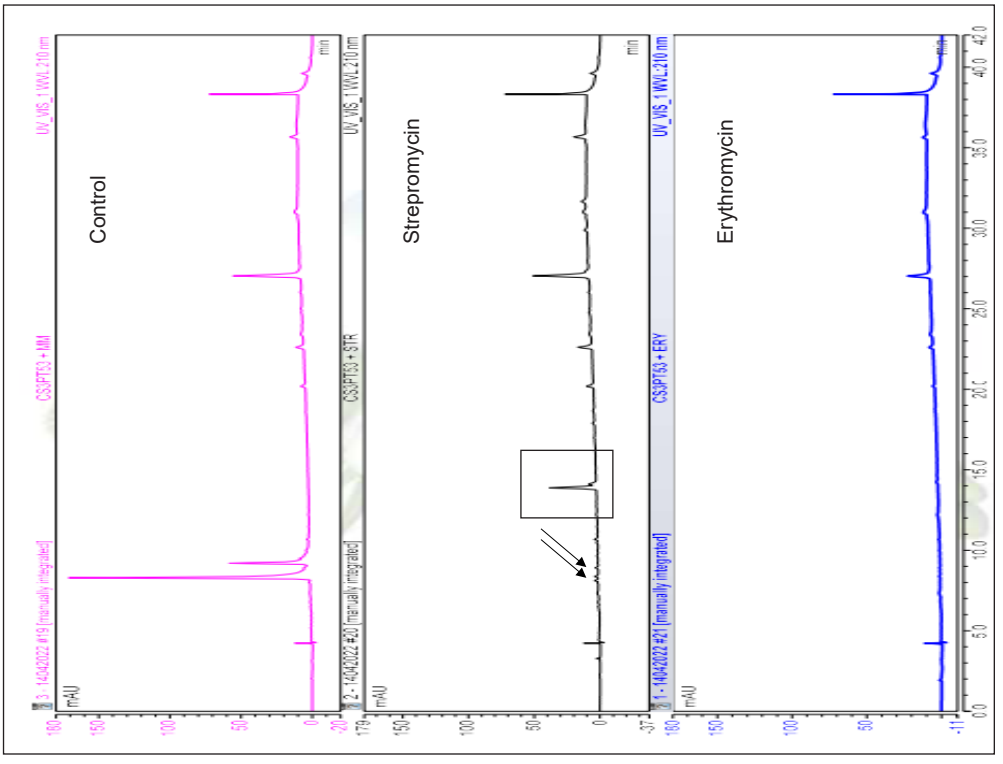



*Figure 4.* HPLC profiles of isolate CS3PT53 extracts at 210 nm

## Antibacterial Activities of Extracts

Besides analysing the secondary metabolite profiles, the differences in antibacterial activity between stimulated and non-stimulated isolates were also investigated using the standard disc diffusion method. The inhibitory activities were further categorised according to their strength: strong for inhibition zone ≥ 20.0 mm, moderate for diameters ranging from 10.0–19.0 mm and weak for diameters < 10.0 mm (Raina et al., 2016). All erythromycin-stimulated crude extracts were excluded since the control extract (cell-free broth with erythromycin alone) inhibited the test bacteria. This was necessary to ensure that the inhibitory activities exerted were purely from the crude extracts, not the elicitors. Table 2 shows the mean diameter of inhibition zones each extract produces on the tested bacteria. Overall, 8 (26.7%) stimulated isolates showed antibacterial properties (6 new activities, 1

Table 2
*Antibacterial activities of stimulated and non-stimulated crude extracts*

| Isolates | Zone of inhibition (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Gram-positive | | | | Gram-negative | | | |
| | ***S. aureus* ATCC 25923** | | ***B. subtilis* ATCC 6633** | | ***E. coli* ATCC 25922** | | ***S.* Typhimurium ATCC 14028** | |
| | NS | S | NS | S | NS | S | NS | S |
| **KMY9** | - | 23.3 ± 0.6*** | - | - | - | - | x | x |
| **PMS2B** | - | - | x | x | - | 11.0 ± 1.0** | x | x |
| **PRS3FI** | - | 10.7 ± 0.6** | - | 8.0 ± 1.0* | - | - | - | - |
| **TBS127** | - | 10.0 ± 1.0** | x | x | - | - | x | x |
| **PGS123** | - | 18.0 ± 1.0** | - | 15.3 ± 0.6** | - | - | - | - |
| **CS1PT57** | 7.3 ± 0.6* | - | 10.0 ± 1.0** | - | - | - | - | - |
| **CS3PT50** | - | - | - | 7.0 ± 1.0* | - | - | x | x |
| **CS3PT53** | 19.3 ± 0.6** | - | x | x | - | - | 11.0 ± 1.0** | 8.3 ± 0.6* |
| **CS3PT5** | 8.7 ± 0.6* | 17.3 ± 0.6** | - | - | - | - | - | - |
| **Positive control** | 31.3 ± 0.6 | | 27.3 ± 0.6 | | 33.0 ± 1.0 | | 29.0 ± 1.0 | |
| **Negative control** | - | | - | | | | - | |

*Notes.* Values are expressed as mean ± standard deviation of three replicates. NS = Non-stimulated isolate; S = Stimulated isolate; Positive control = Ciprofloxacin 5µg/disc; Negative control = 10% DMSO; x = not tested; - = no activity; *** Strong activity (≥ 20.0 mm) ; ** Moderate activity (10.0-19.0 mm); *Weak activity (< 10.0 mm)

improved, and 1 decreased in activities) against at least one test bacteria as compared to the non-stimulated isolates (3 isolates, 10.0%). As expected, most growth inhibitions were seen against Gram-positive as opposed to Gram-negative bacteria due to the differences in cell wall composition between both cells. Moreover, additional protection provided by the outer membrane offers intrinsic protection against external substances, thus increasing the resistance level of Gram-negative bacteria (Breijyeh et al., 2020).

There was a variation in the strength of activities exerted by the extracts, with the majority showing moderate inhibition (60.0%, 9/15). Among the active crude extracts, extract from stimulated KMY9 isolate exhibited the strongest antibacterial activity (23.3 ± 0.6 mm) against *S. aureus* ATCC 25923, while the weakest was CS3PT50 extract (7.0 ± 1.0 mm) against *B. subtilis* ATCC 6633. Four isolates were found to inhibit more than one test bacteria, including PRS3FI, PGS123, CS1PT57, and CS3PT53. Only isolate CS3PT5 showed more than a 2-fold increment (from 8.7 ± 0.6 mm to 17.3 ± 0.6 mm) in the inhibition zone against *S. aureus* ATCC 25923 when supplemented with streptomycin (64 µg/mL), which implied that the corresponding antibacterial compound produced may be enhanced upon antibiotic exposure. Moreover, the HPLC profiles correlate with the increase in growth inhibition as an enhanced peak was detected in the chromatogram (data not shown). This finding is in line with previous reports where the production of actinorhodin, toyocamycin, salinomycin, actinomycin D, tetramycin A, and other relevant antibiotics were significantly boosted using similar techniques (Imai et al., 2015; Shentu et al., 2016; Tanaka et al., 2017).

Apart from that, extract from CS3PT53 showed broad spectrum potential as it inhibited the growth of both *S. aureus* ATCC 25923 (zone inhibition of 19.3 mm ± 0.6 mm) and *S.* Typhimurium ATCC 14028 (zone inhibition of 11.0 mm ± 1.0 mm). However, the inhibition properties displayed were from non-stimulated extract instead of stimulated. A decrease in the inhibition zone was also observed against *S.* Typhimurium ATCC 14028. It might be attributed to the loss of 2 important peaks (shown by the arrows) or reduce in peak intensity at minute 27.0 in the streptomycin-induced chromatogram (Figure 4). In addition, a decrease and loss of activity were also observed in 2 isolates, CS3PT53 and CS1PT57, respectively. Although the HPLC profile of isolate CS3PT53 showed several new peaks when induced with streptomycin (Figure 3), this was not reflected in the antibacterial activities. Nevertheless, these possibly 'new' metabolites may possess other biological properties and should be tested further.

## Molecular Identification of Hit Isolates

All actinomycete isolates displaying antibacterial activities against the test pathogens were sent to a third party (Apical Scientific Sdn. Bhd., Malaysia) for molecular identification. Most of the hit actinomycete isolates belonged to *Streptomyces* (55.6%), followed by

*Norcardia* (22.2%)*, Norcardiopsis,* and *Saccharomonospora* (11.1%, respectively). Detailed results are shown in Table 3.

Table 3
*Molecular identification of active actinomycete isolates*

| No. | Isolates | Closest Cultivated Species | Identity (%) |
|---|---|---|---|
| **1** | KMY9 | *Streptomyces sp.* JF917314.1 | 100 |
| **2** | PMS2B | *Saccharomonospora sp.* JF806667.1 | 100 |
| **3** | PRS3FI | *Nocardiopsis alba* DSM 43377 NR_026340.1 | 100 |
| **4** | TBS127 | *Streptomyces variabilis strain NRRL B-3984* NR_043840.1 | 99 |
| **5** | PGS123 | *Streptomyces carpaticus strain NRRL B-16359*. NR_043814.1 | 99 |
| **6** | CS1PT57 | *Streptomyces atrovirens strain NRRL B-16357* NR_043508.1 | 99 |
| **7** | CS3PT50 | *Nocardia asteroides strain NBRC 15531* NR_041856.1 | 99 |
| **8** | CS3PT53 | *Nocardia huaxiensis strain WCH-YHL-001* NR_181411.1 | 99 |
| **9** | CS3PT5 | *Nocardia amamiensis strain TT 00-78* NR_041531.1 | 99 |

Past studies have reported some identified hit isolates having other biological activities, including antioxidant, antibacterial, antifungal, and anti-biofilm. For example, Janardhan et al. (2014) reported that *N. alba* (PRS3FI) has shown good antioxidant properties. Separately, Quach et al. (2021) described the purification and structural elucidation of *S. variabilis'* metabolites (TBS127) and revealed that the plant-derived bioactive compounds (daidzein, genistein, and isoprunetin) displayed broad-spectrum inhibitory effects against methicillin-resistant *Staphylococcus epidermidis* ATCC 35984 and *S.* Typhimurium ATCC 14028 with MIC values ranging from 16 to 128 µg/mL. In addition, these compounds likewise exhibited significant growth inhibition ($IC_{50}$ < 46 µM) against human lung carcinoma cell line A549. Meanwhile, the extract of *Streptomyces* sp. (similarity closest to *S. atrovirens* NRRL B-16357) isolated from the rhizosphere soil of India's wildlife sanctuary forest showed antimicrobial potential against a wide range of pathogenic bacteria as well as dermatophytes with MIC values < 0.5 µg/mL (Mazumdar et al., 2023). Based on the literature search, no studies have attempted to elicit cryptic secondary metabolites using antibiotics from the present identified isolates.

## CONCLUSION

Overall, this study demonstrated that incorporating antibiotic elicitor at subinhibitory concentration impacts actinomycetes' phenotypic, secondary metabolism, and antibacterial activities from unique environments. The effect can be positive, negative, or a combination. Despite the small percentage differences, metabolic changes were more significant in erythromycin-induced media than in streptomycin. In addition, greater antibacterial activities were found in extracts of stimulated as opposed to non-stimulated isolates with

higher inhibitory against Gram-positive than Gram-negative bacteria. The data suggests that antibiotic elicitors can potentially elicit new antibacterial metabolites in actinomycetes and can be exploited further for other biological activities. Further investigation is underway to optimise the production of bioactive compounds and identify the metabolite responsible for the biological activity.

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