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ENDOPHYTIC ACTINOMYCETES FROM MANGROVE PLANT *Avicennia marina* IN QUANG NINH PROVINCE, VIETNAM: DISTRIBUTION, CYTOTOXICITY, AND ANTIOXIDANT ACTIVITIES

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ABSTRACT

Mangrove endophytes have recently gained considerable attention due to their diversity and abundance of novel bioactive secondary metabolites. Despite the fact that *Streptomyces* species are producers of more than 75% of commercialized antibiotics, *Streptomyces* associated with the extremely widespread mangrove plant *Avicennia marina* remain poorly characterized. In this study, nine actinomycetes were isolated from *A. marina* growing in a mangrove forest, as yet unexplored, of Quang Ninh province, Vietnam. Phylogenetic analysis of actinomycetes-specific 16S rRNA sequences indicated that they were subjected to five *Streptomyces* species including *Streptomyces cacaoi*, *Streptomyces californicus*, *Streptomyces enissocaesillis*, *Streptomyces coelicoflavus*, and *Streptomyces variabilis*, which have not been previously reported in mangrove plants. Among them, *S. cacaoi* AM1 showed strong inhibition effects against six tested pathogenic bacteria with inhibitory zones ranging from 7.5−22.3 mm. Using standard 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, 100 µg/mL ethyl acetate extract of AM1 showed potent cytotoxicity against breast cancer MCF-7 and lung cancer A549 cell lines with cell viability of 16.5 ± 1.28% and 17.69 ± 2.3%, respectively. As for antioxidant activities, AM1 extract exhibited strong antioxidant activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (92.4 ± 0.004%) and superoxide anion radical (42.4 ± 0.019%), which were positively correlated to high polyphenol (84.3 ± 3.4 µg GAE/g FW) and flavonoid (34.9 ± 4.8 µg QE/g FW) contents. These findings indicated that *S. cacaoi* AM1 could be a promising reservoir of antibacterial, anticancer, and antioxidant agents. This is the first report of mangrove endophytic *Streptomyces* derived from *A. marina.*

Keywords: Antibacterial, anticancer, antioxidant, *Avicennia marina*, mangrove, *Streptomyces. cacaoi.*

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INTRODUCTION

Production of reactive oxygen species (ROS) including hydroxyl radical, superoxide anion, hydrogen peroxide, and singlet oxygen is considered by-products of aerobic metabolism in plants and humans (Yang et al., 2015). However, excessive generation of ROS can disrupt the integrity of membrane lipids, proteins, and DNA, leading to several chronic diseases, such as diabetes, atherosclerosis, rheumatoid arthritis, cardiovascular disease, chronic inflammation, ageing, and especially cancer (Griffiths et al., 2016). Antioxidant radical scavenging is necessary to inhibit or delay cellular damages. Of note, various antioxidant compounds also have anticancer, antimicrobial, and inflammatory activities (Dholakiya et al., 2017; da Silva et al., 2020). Since synthetic antioxidants were terminated in the market due to their carcinogenicity and toxicity (Griffiths et al., 2016), special attention has been paid to natural products from endophytes.

Great interest has been placed on exploiting mangrove endophytic microorganisms among which actinomycetes have gained the spotlight. Endophytic actinomycetes residing inside plant tissues without any discernible infectious symptoms are known to produce novel and the same natural bioactive compounds for which the plant is known (Jiang et al., 2018; Wang et al., 2019; Singh & Dubey, 2020). As compared to actinomycetes derived from medicinal plants, reports related to mangrove endophytic actinomycetes dominated by the genus *Streptomyces* and their secondary metabolites are still scarce (Jiang et al., 2018). A previous study showed that *Streptomyces* sp. HKI0595 from mangrove plant *Kandelia candel* (L.) Druce produced three novel compounds including xiamycin, indosespene, and sespenine that inhibited methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* (Ding et al., 2011). Four novel cyclopentene derivatives were extracted from *Streptomyces* sp. GT-20026114 isolated from *Aegiceras corniculatum* (L.) Blanco, however, antimicrobial, anticancer, and antiviral activities were not detected (Wang et al., 2010). In contrast to antimicrobial activity, antioxidant and anticancer properties have not been fully explored in mangrove endophytic actinomycetes, raising a great chance of finding novel bioactive compounds.

The literature survey shows that *Avicennia marina* var. *rumphiana* (Hallier. f.) Bakh. is rich in phenolic and flavonoid compounds that can inhibit pathogens, cancer cells, and free radicals (Momtazi-Borojeni et al., 2013; Okla et al., 2021). However, endophytic actinomycetes associated with *A. marina* and their biological potential in medical and therapeutic applications have not been revealed yet. Hence, this study was performed to isolate and screen endophytic actinomycetes with antibacterial, anticancer, and antioxidant activities from *A. marina* collected from Quang Ninh mangrove forests, Northern Vietnam where studies on endophytes are scarce. To our knowledge, this is the first report of endophytic actinomycetes isolated from *A. marina* that provides the basis for further investigation of novel bioactive metabolites with medical and therapeutic applications.

MATERIALS AND METHODS

Sampling and isolation of endophytic actinomycetes

Mangrove plant *A. marina* was collected from different sites in Quang Ninh mangrove forest, Northern Vietnam (21.0064^{o}N, 107.2925^{o}E). Samples from the healthy plant were divided into 3 parts including leaves, stems, and roots then placed in sealed plastic bags and transferred to the laboratory of the Institute of Biotechnology, Vietnam Academy of Science and Technology. The plant voucher specimens were then identified as *A. marina* by the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The plant samples were washed with running tap water and air-dried under a laminar flow hood, which were subsequently subjected to surface sterilization procedure as previously described (Salam et

al., 2017). In brief, the samples were washed in 5% (v/v) NaOCl for 4 min, washed in 2.5% (w/v) $Na_2S_2O_3$ for 10 min, and then rinsed in 75% ethanol for 5 min. In the last step, sterile water and 10% (w/v) $NaHCO_3$ were used to complete the surface sterilization procedure. The surface-sterilized samples were crushed and then spread onto 3 selective isolation media including humic acid-vitamin B, raffinose-histidine, and sodium succinate-asparagine media supplemented with 50 mg/mL nystatin, 25 mg/mL $K_2Cr_2O_7$, and 25 mg/mL nalidixic acid. After 4 weeks, colonies grown on these isolation media were streaked out several times on the YIM38 medium (Wang et al., 2019). The pure isolates were preserved in 15% (v/v) glycerol at -80 ^oC.

Molecular identification of endophytic isolates

Genomic DNA of each isolate was extracted using the G-spin™ Total DNA Extraction Mini Kit (Intron Bio) following the manufacturer's protocol. PCR amplification for the 16S rRNA gene from the extracted DNA samples was performed using the primer pair 27F (5'-TAACACATGCAAGTCGAA CG-3') and 1429R (5'-GGTGTGACGGGCG GTGTGTA-3') (Vu et al., 2020). Amplified PCR products were purified with a DNA purification kit (Promega, Madision, USA) and then sequenced by First BASE Laboratories Sdn. Bhd (Malaysia). The resulting 16S rRNA genes were compared with those available on GenBank (NCBI) (http://www.ncbi.nlm.nih.gov/) and the EzTaxon server (http://www.eztaxon.org/). The phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap using Kimura 2-parameter distances in MEGA v7.0 (Kumar et al., 2016). *Nocardia farcinica* ATCC 3318^T (NR 115831) was used as the outgroup branch.

Antibacterial potential of endophytes

A panel of six pathogenic bacteria including *Enterobacter aerogenes* ATCC13048, *Staphylococcus epidermidis* ATCC 35984, *Escherichia coli* ATCC 11105, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus cereus* ATCC 11778, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC14028 was used to test the antibacterial activity of all endophytic strains. All endophytic strains were cultivated in YIM38 medium for a period of 14 days at 30 ^oC. Antibacterial activity was evaluated following the good diffusion assay (Balouiri et al., 2016). The experiment was performed in triplicate and the results were represented by a heatmap (Williams et al., 2019).

Preparation of ethyl acetate crude extracts

The most promising strain was inoculated in a YIM38 medium and incubated for 14 days at 30 ^oC under shaking conditions. The mycelium and filtrate of the culture broth were filtered to separate each other. The cell-free culture supernatant was collected and mixed with an equal volume of ethyl acetate (Dholakiya et al., 2017). The crude extract was obtained using a rotary flash evaporator. The dried crude extract was dissolved in 10% (v/v) dimethyl sulfoxide (DMSO) and 70% (v/v) ethanol for the cytotoxic and antioxidant experiments, respectively.

Cytotoxicity assay of AM1 crude extract

The cytotoxic activity of the crude extract was examined towards breast cancer MCF-7 and lung cancer A549 cell lines using standard 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (Quach et al., 2021). The cell viability was determined based on the presence of purple formazan crystals that emerged from the reduction of MTT dye, which was measured spectrophotometrically at 570 nm using the microplate reader. Camptothecin and DMSO were used as positive and negative controls, respectively.

Antioxidant activities of AM1 crude extract

About 100 µL of the extract solution in the concentration of 0–1.6 mg/mL was reacted to 100 µL of 0.1 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution, followed by incubation at room temperature for 30 min in darkness. The absorbance was measured at

517 nm against an equal amount of DPPH. The percentage of DPPH scavenging activity was calculated using the following formula (Kadaikunnan et al., 2015):

$$\text{Scavenging activity}(\%) = \left[1 - \frac{(A_{sample} - A_0)}{A_{blank}}\right] \times 100$$

Where: A_{sample} is the absorbance of the reaction mixture; A_0 is the absorbance of 70% ethanol and crude extract; A_{blank} is the absorbance of 70% ethanol.

The free superoxide radical scavenging capacity was evaluated as described previously (Yang et al., 2015). About 200 µL of the crude extract (0–1.6 mg/mL) was mixed with 900 µL of 0.05 M Tris–HCl (pH 8.2) and 80 µL of 2.5 mM pyrogallol, followed by incubation at room temperature for 5 min. The absorbance at 299 nm was measured and the percentage of superoxide radical scavenging activity was calculated as:

$$\text{Scavenging activity}(\%) = \frac{(A_{sample} - A_0)}{A_{blank}} \times 100$$

Where: A_{sample} is the absorbance of the reaction mixture; A_{blank} is the absorbance of the blank (70% ethanol instead of sample and reagents).

For the reducing power activity, different concentrations of extract (0-1.6 mg/mL) were made with a final volume of 300 µL and mixed with 300 µL of 0.2 M sodium phosphate buffer pH 7.3. The reaction was started by the addition of 1.5 mL of 1% (w/v) $K_3Fe(CN)_6$ and kept for 25 min in the dark. About 300 µL of 12% (w/v) trichloroacetic acid was added to the reaction mixture. Next, about 1 mL of supernatant was mixed with 0.5 mL of 0.2% (w/v) $FeCl_3$. The reducing power was calculated as follows (Rajoka et al., 2019): Reducing ability = A_{sample}−A, where A was the absorbance of mixture containing deionized water instead of 0.2% $FeCl_3$ (w/v).

Total polyphenol and flavonoid contents

For polyphenol determination, the mixture containing 20 µL of 70% ethanol extract and 100 µL of Follin-Ciocalteu reagent was incubated at room temperature for 5 min, followed by the addition of 80 µL of 4% (w/v) sodium carbonate to stop the reaction (da Silva et al., 2020). The sample was spectrophotometrically measured at 765 nm using a microplate reader. Total polyphenol was expressed as µg of the gallic acid equivalent/g of the extract (µg GAE/g FW). In flavonoid assay, about 30 µL of 70% ethanol extract was added to the mixture consisting of 10 µL of 5% (w/v) $NaNO_2$, 10 µL of 10% (w/v) $AlCl_3$, 60 µL of 1M NaOH, and 120 µL of distilled water (Tang et al., 2021). The reaction was kept at room temperature for 30 min. Absorbance at 510 nm was measured by using a microplate reader. Flavonoid content was measured as µg of the quercetin equivalent/g of the extract (µg QE/g FW). All experiments were performed in triplicates.

RESULTS AND DISCUSSION

Distribution of endophytic actinomycetes

A total of nine endophytic colonies with different morphological characteristics were isolated from *A. marina* (Fig. 1A). The appearance of hyphae, slow growth, and production of spores and pigments as typical characteristics of actinomycetes were observed for all isolates. Actinomycetes were found in all the plant tissues investigated, suggesting the ability of actinomycetes to occupy different parts throughout the plant. The majority of endophytic isolates were recovered from roots (66.7%), followed by

stems (22.2%), and leaves (11.1%) (Fig. 1B). This was in agreement with previous studies showing the abundance of endophytic actinomycetes with the highest percentage in roots (Taechowisan et al., 2003; Musa et al., 2020). In contrast, other reports have proved that most actinomycetes were from stems or leaves compared to roots (Salam et al., 2017; Jiang et al., 2018; Vu et al., 2020). This holds true that the distribution of endophytes varies depending on many factors such as host plant, geographic location, and isolation approach.

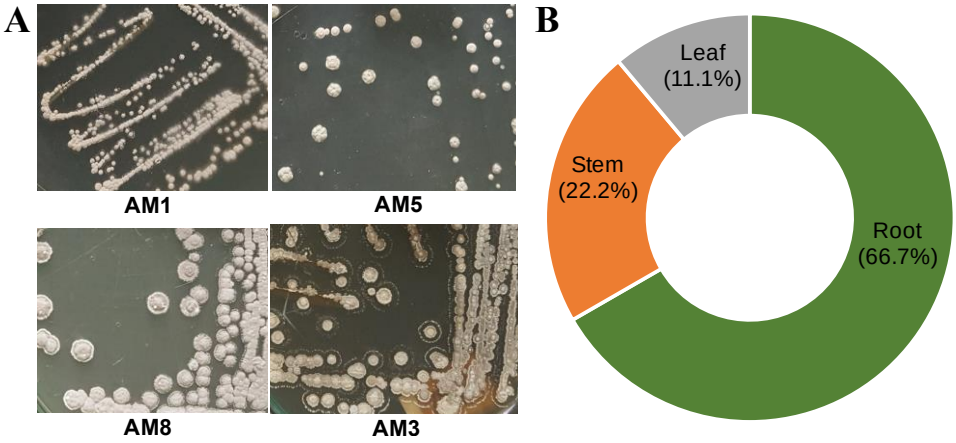

Figure 1. Morphological appearance of representative isolates (A) and distribution of endophytic actinomycetes from *Avicennia marina* according to plant organs (B)

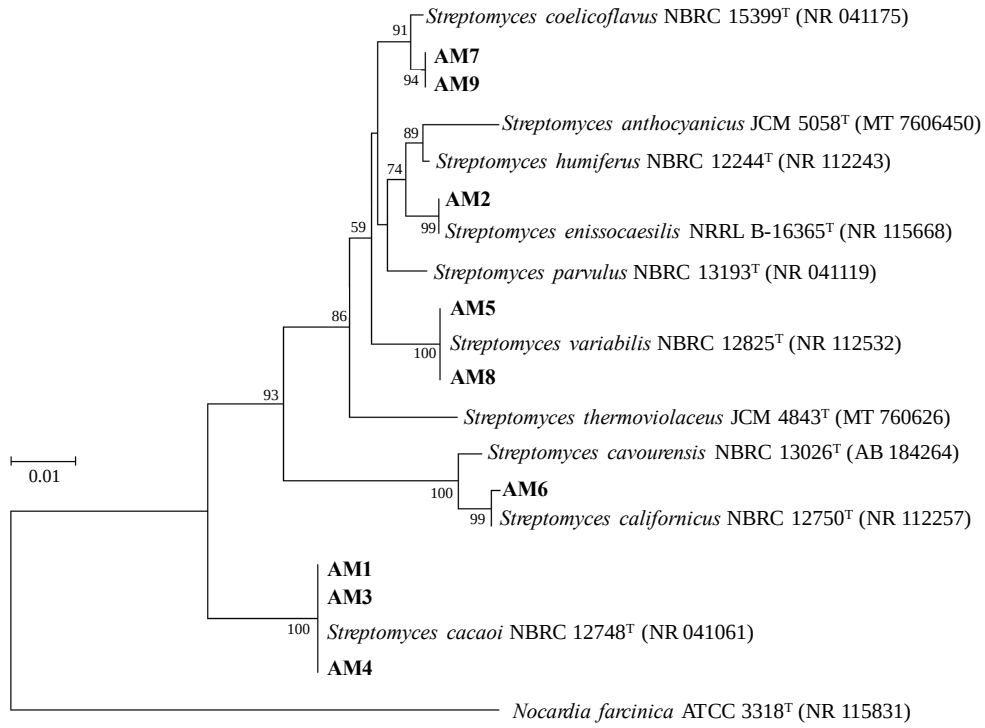

Figure 2. Neighbor-joining tree of the 16S rRNA sequences showing the relationship between endophytic actinomycetes isolated from *A. marina* and other closely related species belonging to the genus *Streptomyces*

Analysis of 16S rRNA gene sequences showed that nine isolates had a high similarity of 99.6 to 100% with the type strains belonging to *Streptomyces* species. In addition, the neighbor-joining phylogenetic tree was constructed to show that all nine isolates were grouped into five *Streptomyces* species including *Streptomyces cacaoi* (AM1, AM3, AM4), *Streptomyces californicus* (AM6), *Streptomyces enissocaesillis* (AM2), *Streptomyces coelicoflavus* (AM7, AM9) and *Streptomyces variabilis* (AM5, AM8) (Fig. 2). The 16S rRNA gene sequences of AM1–AM9 were deposited in GenBank (NCBI) under accession numbers from ON413776–ON413784, respectively.

To date, *S. cacaoi* was originally recovered from the cacao bean and marine, but not from mangrove plants (Lanoot et al., 2002; Khan et al., 2019). *Streptomyces californicus* ADR1 was reported as an endophytic strain of the medicinal plant *Datura metel* (Singh & Dubey, 2020). To the best of our knowledge, this is the first study reporting endophytic strains of *cacaoi*, *Streptomyces californicus*, *Streptomyces enissocaesillis*, *Streptomyces coelicoflavus* and *Streptomyces variabilis* from mangrove plants.

Screening of antibacterial activity of bioactive actinomycetes

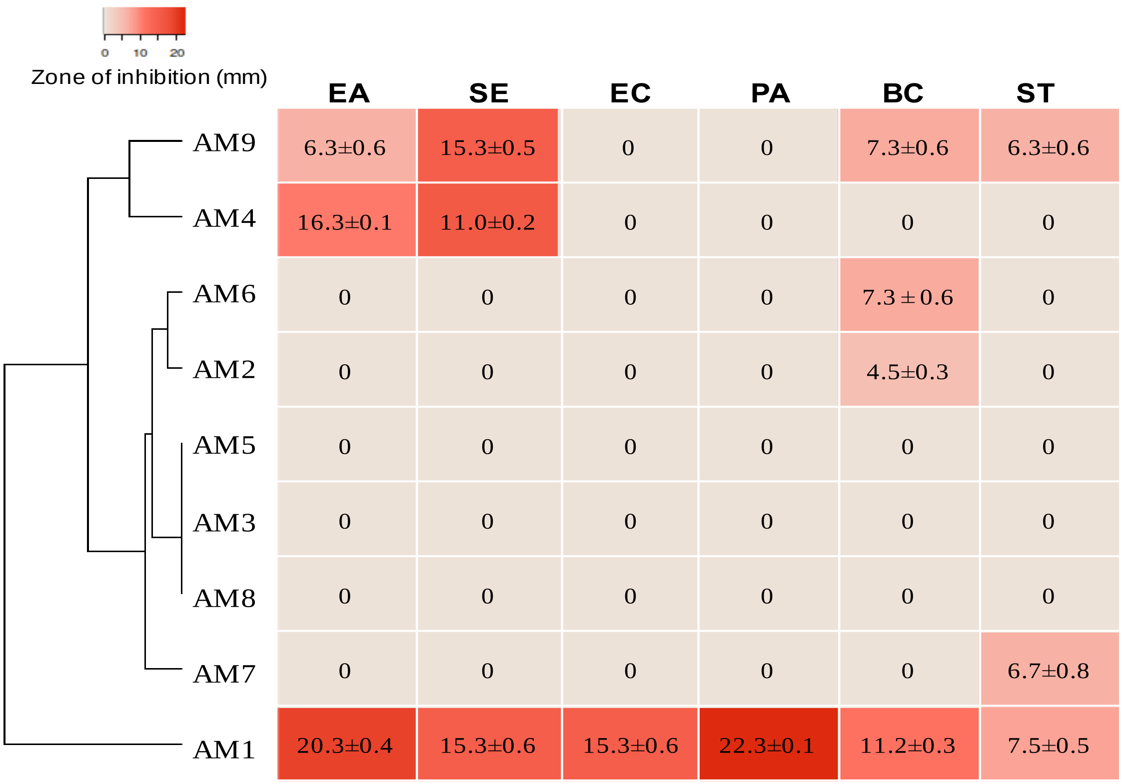

Figure 3. Heatmap showing the antibacterial potential of nine actinomycetes associated with *A. marina*. Where, EA: *Enterobacter aerogenes* ATCC13048; SE: *Staphylococcus epidermidis* ATCC 35984; EC: *Escherichia coli* ATCC 11105; PA: *Pseudomonas aeruginosa* ATCC 9027; BC: *Bacillus cereus* ATCC 11778; ST: *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC14028

Nine strains were evaluated for their antibacterial activities against six bacterial pathogens. Of the nine strains, six strains (66.7%) showed antibacterial activities

against at least one pathogen (Fig. 3). A previous study indicated that only 45% of *Streptomyces* strains derived from mangrove soil in China displayed antibacterial activity against pathogenic bacteria (Li et al., 2019). It is believed that microbes from the extreme environment like the mangroves possess the potential to synthesize novel compounds with distinct biological activities (Sangkanu et al., 2017; Pavan Kumar et al., 2018).

Among six bioactive strains, AM9 and AM4 had similar antibacterial properties against *E. aerogenes* and *S. epidermidis* with a moderate zone of inhibition of less than 17 mm. AM9 also displayed antagonist effects on *B. cereus* ATCC 11778 and *S.* Typhimurium ATCC14028. The weak inhibitory effect against only one pathogen was found in strains AM2, AM6, and AM7. Notably, *S. cacaoi* AM1 presented antagonistic activity against all tested pathogens with the strongest effect against *P. aeruginosa* ATCC 9027 (22.3 ± 0.1 mm), *E. aerogenes* ATCC13048 (20.3 ± 0.4 mm), *S. epidermidis* ATCC 35984 (15.3 ± 0.6 mm), *E. coli* ATCC 11105 (15.3 ± 0.6 mm), *B. cereus* ATCC 11778 (11.2 ± 0.3 mm), and *S.* Typhimurium ATCC14028 (7.5 ± 0.5 mm) (Fig. 3). In support of this result, the endophytic *S. cacaoi* subsp. *cacaoi* NBRC 12748^T was able to produce pentaminomycin C which was active against Gram-positive bacteria (Kaweewan et al., 2020). Marine *S. cacaoi* had antimicrobial activity against vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *S. aureus* (Khan et al., 2019). It is evident that *Streptomyces* genomes possess 25–70 biosynthetic gene clusters, in which most biosynthetic gene clusters remain inactivated and are only induced under a specific condition (Belknap et al., 2020). Hence, *S. cacaoi* AM1 could be a promising candidate to further exploit new antibacterial agents.

Evaluation of the cytotoxic activity of the *S. cacaoi* AM1 extract

The cytotoxic activity of the *S. cacaoi* AM1 strain was evaluated using its crude extract on two human cancer cell lines A549 and MCF-7. Exposure to 100 μg/mL extract resulted in 17.7% and 16.5% cell viability of A549 and MCF-7, respectively (Fig. 4A). Different to this result, treatment with 200 μg/mL of crude extract of mangrove soil-derived *S. cacaoi* M20 led to 49% growth inhibition of MCF-7 (Janaki, 2019). In addition, polyether compounds including K41 A and polyether-type inophores extracted from marine-derived *S. cacaoi* strongly inhibited the growth of A549 (Khan et al., 2019). It was speculated that growth inhibition of cancer cells when treated with crude extract may be linked to inhibition of autophagy signified by the build-up of autophagy markers LC3-II and p62 as well as the p53 tumor suppressor protein status leading to apoptosis (Petitjean et al., 2007; Khan et al., 2019).

Antioxidant activities of the AM1 extract

Since antioxidant agents have been linked to reduced risk of developing chronic diseases and cancer (Griffiths et al., 2016), the antioxidant potential of AM1 extract was evaluated using three methods, namely DPPH radical scavenging assay, superoxide anion radical scavenging assay, and reducing power assay. Under treatment with 1.6 mg/mL extract, superoxide radical scavenging activity was recorded at 42.4 ± 0.019% (Fig. 4b). At the same extract concentration, DPPH free radical scavenging activity reached the highest level (92.4 ± 0.004%), which was comparable to ascorbic acid (95.4 ± 0.211%). In addition, the reducing power assay assessing the reduction potential of AM1 to convert ferric ion Fe^{3+} to Fe^{2+} showed weak chelation activity with OD_{700nm} of 0.255 ± 0.018 at 1.6 mg/mL extract (Fig. 4c).

An intriguing research demonstrated that pentaminomycin C and D isolated from *Streptomyces* sp. GG23, a strain closely related to *S. cacaoi*, showed a cytoprotective effect against oxidative stress induced by menadione (Kaweewan et al., 2020). In addition, pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro- purified from mangrove *S. mangrovisoli* sp. MUSC 149^T exhibited scavenging activity of 36.5% against DPPH at 2.0 mg/mL (Ser et al., 2015), which was 2.5-

fold lower than that of AM1 extract. This shred of evidence suggested *S. cacaoi* AM1 likely produced known and unknown metabolites with antioxidant properties.

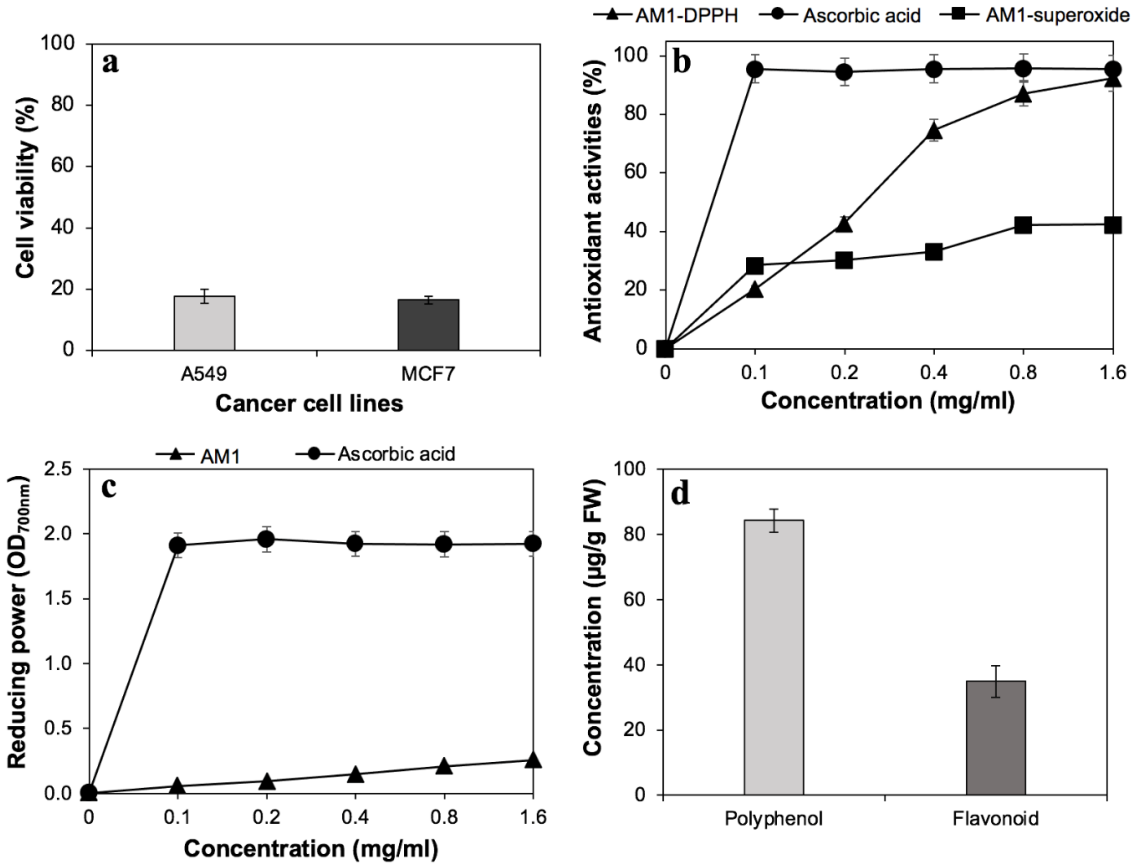

Figure 4. Biological activities of ethyl acetate crude extract of *Streptomyces cacaoi* AM1. Cytotoxicity (a), antioxidant activities (b, c), and total flavonoid and polyphenolic contents (d) of *Streptomyces cacaoi* AM1 extract

Determination of the total polyphenol and flavonoid contentss

In supporting the antioxidant activities, total polyphenol and flavonoid contents were determined from the *S. cacaoi* AM1 crude extract. AM1 extract contained 84.3 ± 3.4 µg GAE/g FW total polyphenol and 34.9 ± 4.8 µg QE/g FW flavonoid, confirming the ability of AM1 to produce plant-derived metabolites (Fig. 4d). Polyphenols and flavonoids are natural compounds of plant origin that possess extensive pharmacological activities such as anti-inflammatory, anticancer, antibacterial and antioxidant (Wang et al., 2020). In agreement with our findings, phenolic compounds such as phenol,2,2-methylenebis [6-(1,1-dimethylethyl)-4-methyl-; phenol,2,5-bis (1,1-dimethylethyl)- and phenol, 2,20-methylenebis [6-(1,1dimethylethyl)-4-methyl-] previously detected from mangrove *Streptomyces* sp. MUM292 and MUM212 showed lower DPPH scavenging activities as compared to AM1 extract (Tan et al., 2018). Although *Streptomyces cellulosae* TES17 from *Camellia sinensis* produced higher total polyphenols and flavonoids than the AM1 extract, antioxidant activity against DPPH was much lower than that of AM1 (Rani et al., 2018). These results suggest that polyphenols

and flavonoids might not entirely be responsible for the potent antioxidant properties of AM1 extract. Further detailed analysis of secondary metabolites in pure form of *S. cacaoi* AM1 will be an interesting subject for future investigations.

CONCLUSION

In summary, this is the first study reporting endophytic actinomycetes isolated from mangrove plant *A. marina* that were collected from Quang Ninh mangrove forest, Northern Vietnam. Nine *Streptomyces* strains were isolated, of which 66.7% of the actinomycetes were from roots, 22.2% from stems, and 11.1% from leaves. Among them, *S. cacaoi* AM1 was the only strain that displayed broad-spectrum antibacterial activity against all tested pathogens. Moreover, the extract of AM1 was able to inhibit the growth of cancer cell lines A549 and MCF-7. Polyphenol and flavonoids in the extract could contribute to the antioxidant activities against free radicals including DPPH, superoxide, and metal ions chelation. These findings demonstrated that *S. cacaoi* AM1 could be a promising candidate for exploiting novel bioactive metabolites potentially used for therapeutical applications.

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