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Original Article

An Investigation on Antibacterial and Antifungal Activities of Actinomycetes Associated with 17 Medicinal Plants Collected in Northern Vietnam

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Abstract: Actinomycetes associated with plants are those that live on the surface, inside, or outside the plants, and have a reciprocal relationship with the plants. These actinomycetes have recently been found to possess many beneficial activities. The Northwest region of Vietnam is home to various valuable medicinal plants, of which the health-healing compounds, the microorganisms, particularly actinomycetes, are a promising research subject. In this study, we screened 136 endophytic and surface-associated actinomycetous strains isolated from 17 medicinal plants collected in the Northwest region of Vietnam for their antimicrobial activities against *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Aspergillus fumigatus*, and *Aspergillus flavus*. The results showed that 5 strains were antagonistic to *Escherichia coli*, 22 strains were antagonistic to *Bacillus subtilis*, 3 strains were antagonistic to all the three test fungi, 6 strains were antagonistic to more than one test microorganism, and 2 strains were antagonistic to all test microorganisms. Three actinomycetous strains (AL12.3, AT4.1, and AS10.10) with outstanding activities were identified as *Streptomyces* spp. The optimal conditions for their growth were determined to be as follows: ISP4 medium with potato extract, temperature at 30 °C, culture duration of 6-7 days for strain AL12.3, and 3-4 days for strains AT4.1 and AS10.10. A preliminary experiment in this study showed that when cultured on a medium supplemented with the aqueous extract of its cognate plant, strain AT4.1 displayed an enhanced antibacterial activity against *E. coli* and *B. subtilis*, compared to culture conditions without the extract.

Keywords: Antibacterial activity, antifungal activity, endophytic actinomycetes, medicinal plants.

1. Introduction

Among microorganisms, actinomycetes are known to produce a wide range of secondary metabolites, including antibiotics, enzymes, and anticancer compounds. Many studies have reported on the biological activity of actinomycetes isolated from soil in the rhizosphere of medicinal plants as well as endophytic actinomycetes isolated from plant tissues. Research on endophytic actinomycetes isolated from medicinal plants has discovered

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their diverse biological activities such as antimicrobial, antifungal, antiviral, anticancer, antioxidant, and insecticidal activities [1-6]. This implies many important applications in agriculture, medicine, and pharmaceuticals. In addition, surface-associated actinomycetes in soil in the rhizosphere of medicinal plants are also a subject of interest. Actinomycetes isolated from soil in the rhizosphere of medicinal plants have been found to also have antimicrobial activities, enzyme production, and plant growth-promoting properties [7-11].

With a sense of awareness that the resources of medicinal plants in Vietnam are very diverse and abundant but have not received adequate attention in research, this study aims to investigate the potential of actinomycetes associated with medicinal plants. Specifically, we screened endophytic and surface-associated actinomycetes isolated from medicinal plants in the Northwest region of Vietnam for their antibacterial and antifungal activities. Only these two activities were studied within the scope of this study due to limited study time and the aim of finding new biocontrol agents for plant protection. The interesting strains will be further studied in terms of their biological characteristics and the effects of host plant extracts on their biological activities.

2. Experimental

2.1. Media

Media used in this study and their respective compositions (per liter) are as follows: Luria-Bertani (LB): 10 g tryptone, 5 g yeast extract, 10 g NaCl [12]. Potato-Dextrose (PD): filtrate of 500 mL of water containing 300 g of boiled finely diced potatoes, 20 g glucose [13]. ISP4: 10 g soluble starch, 1 g K_2HPO_4, 1 g $MgSO_4$, 1 g NaCl, 2 g $(NH_4)_2SO_4$, 2 g $CaCO_3$, 5g peptone, 50 µL of trace element solution (containing 29.5 g/L $FeSO_4.4H_2O$, 20 g/L $MnCl_2$ C, 36.7 g/L $ZnSO_4.7H_2O$), the boiled water extract from 200 g potato (if needed) [14]. YS: 2 g yeast extract, 10 g soluble starch. Gauze No. 1: 20.0 g soluble starch, 1.0 g KNO_3, 0.5 g NaCl, 0.5 g $MgSO_4.7H_2O$, 0.5 g K_2HPO_4, 10.0 g $FeSO_4.7H_2O$ [14]. To prepare an agar medium, 15-20 g agar was added per litter.

2.2. Study Objects

In our previous study, 17 medicinal plants were collected from the Northwest region of Vietnam, identified and classified, following standard procedures as previously described [15]. They include: *Caesalpinia sappan* L (KT01), *Equisetum ramosissimum* (KT04), *Glochidion eriocarpum* Champ.exBenth (KT05), *Anisomeles indica* (L.) Kuntze (KT06), *Clerodendrum cyrtophyllum* Tucz (KT07), *Pericampylus glaucus* (Lam.) Merr (KT08), *Mahonia bealei* (Fortune) Pynaert (KT09), *Ficus semicordata* Buch-Ham.ex Sm (KT10), *Gnetum montanum* Markgr (KT11), *Tacca chantrieri* André (KT12), *Crinum asiaticum*L (KT13), *Mallotus barbatus* Müll.Arg (KT14), *Hedyotis capitellata* Wall.exG.Don (KT16), *Stixis suaveolens* (KT17), *Cymbidium aloifolium* (L.) Sw (KT18), *Croton kongensis* Gagnep (KT19), and *Tinospora sinensis* (Lour.) Merr. (KT20) [15].

136 endophytic and epiphytic actinomycetous strains previously isolated from those 17 medicinal plants were used for antimicrobial activity screening in this study. The list of the strains was presented in Table 1.

2.3. Standard Microorganisms for Antimicrobial Tests

Bacteria: *Escherichia coli* VTCC12272 was provided by the Institute of Microbiology and Biotechnology, Vietnam National University; *Bacillus subtilis* VS was provided by the Laboratory of Microbiology, Faculty of Biology, University of Science, Vietnam National University.

For the antibacterial tests, suspensions the standard bacterial strains (containing ~10^9 CFU/mL) were prepared by shaking incubation in LB medium at 37 °C, 120 rpm overnight.

Fungi: *Candida albicans* JCM2070 and *Aspergillus fumigatus* VTCC015 were provided by the Genomics Laboratory, Faculty of Biology, University of Science, Vietnam

National University; *Aspergillus flavus* VTCC013 was provided by the Institute of Microbiology and Biotechnology, Vietnam National University.

For the antifungal tests, suspensions of the standard fungal strains were prepared by shaking incubation in PD medium at 30 ºC, 120 rpm, for 2-3 days.

2.4. Assessment of Antibacterial and Antifungal Activities

The agar diffusion method: Based on the antagonistic activity of microorganisms, this method is used in experiment ts for screening for actinomycetes having antimicrobial activities and assessing the effects of various factors on those activities [16]. Specifically, each strain of interest was cultured on ISP4 medium at 30 °C for 5-10 days, and then an agar plug was cut out from the culture plate and placed on the surface of a LB agar plate, which was already spread with 100 uL of the suspension of the standard bacterial strain prepared as described above, or a PD agar plate already spread with 100 uL of the suspension of the standard fungal strain prepared as described above. The agar plates were incubated at 4 °C for 2-4 hours and then cultured at 37 °C for the LB plates and 30 °C for the PD plates. Inhibition zones were observed after 24 hours (for antibacterial tests) or 2-3 days (for antifungal tests). The inhibition level (high, medium, or low) was judged according to some previous studies [19].

Table 1. The actinomycete strains used in this study in relation to their respective medicinal host plants

Label/code	Scientific names (Vietnamese name)	Endophytic actinomycetes		Surface-associated actinomycetes	
		Quantity	Strains	Quantity	Strains
KT01	*Caesalpinia sappan* L (To moc)	-	-	6	AS1.1, AS1.2, AS1.3, AS1.4, AS1.5, AS1.6
KT04	*Equisetum ramosissimum* (Co thap but xoe)	6	AR4.1, AR4.2, AR4.3, AR4.4, AT4.1, AT4.2	11	AS4.1, AS4.2, AS4.3, AS4.4, AS4.5, AS4.6, AS4.7, AS4.8, AS4.9, AS4.10, AS4.11
KT05	*Glochidion eriocarpum* Champ. ex Benth (Bon bot)	5	AL5.3, AL5.4, AL5.5, AL5.6, AL5.8	5	AS5.1, AS5.2, AS5.3, AS5.4. AS5.5
KT06	*Anisomeles indica* (L.) Kuntze (Phong phong thao)	1	AL6.2.2	11	AS6.1, AS6.2, AS6.3, AS6.4, AS6.5, AS6.6, AS6.7, AS6.8, AS6.9, AS6.10, AS6.11
KT07	*Clerodendrum cyrtophyllum* Tucz (Bo may)	-	-	2	AS7.1, AS7.2

KT08	*Pericampylus glaucus* (Lam.) Merr (Tiet de la day)	-	-	4	AS8.1, AS8.2, AS8.3, AS8.4
KT09	*Mahonia bealei* (Fortune) Pynaert (Hoang lien)	-	-	7	AS9.1, AS9.2, AS9.3, AS9.4, AS9.5, AS9.6, AS9.7
KT10	*Ficus semicordata* Buch.-Ham. ex Sm (Da la lech)	-	-	10	AS10.1, AS10.2, AS10.3, AS10.4, AS10.5, AS10.6. AS10.7, AS10.8, AS10.9, AS10.10
KT11	*Gnetum montanum* Markgr (Day gam nui)	-	-	8	AS11.1, AS11.2, AS11.3, AS11.4, AS11.5, AS11.6, AS11.7, AS11.8
KT12	*Tacca chantrieri* André (Rau hum)	4	AL12.3, ABAS.3, AR12.1.2, AR12.6.1	9	AS12.1, AS12.2, AS12.3, AS12.4, AS12.5, AS12.6, AS12.7, AS12.8, AS12.9
KT13	*Crinum asiaticum* L (Nang hoa trang)	5	AR13.1, AR13.4.1, AR13.4.2, AR13.4.3, AR13.6	8	AS13.1, AS13.2, AS13.3, AS13.4, AS13.5, AS13.6, AS13.7, AS13.8
KT14	*Mallotus barbatus* Müll.Arg (Bum bup)	-	-	2	AS14.1, AS14.2
KT16	*Hedyotis capitellata* Wall. ex G.Don (Da cam)	-	-	7	AS16.1, AS16.2, AS16.3, AS16.4, AS16.5, AS16.6, AS16.7

KT17	*Stixis suaveolens* (Trung cuoc)	5	AR17.1, AR17.2, AR17.3, AR17.4, AT17.1	-	-
KT18	*Cymbidium aloifolium* (L.) Sw (Lan kiem)	-	-	-	-
KT19	*Croton kongensis* Gagnep (Kho sam)	-	-	5	AS19.1, AS19.2, AS19.3, AS14.4, AS19.5
KT20	*Tinospora sinensis* (Lour.) Merr. *(Day dauxuong)*	9	AT20.1, AT20.2, AT20.3, AT20.4, AT20.5, AT20.6, AT20.7, AR20.1, AR20.2	6	AS20.1, AS20.2, AS20.3, AS20.4, AS20.5, AS20.6

2.5. Identification of Actinomycetes

16S rDNA sequence analysis: The actinomycetous strains with remarkable inhibition activities were further identified firstly by their colony and spore chain morphology (observed under a microscope) and then by the analysis of their 16S ribosomal DNA (rDNA) sequences. The strains were cultured on YS medium at 30 °C for 5-10 days to observe their morphology and describe their characteristics. Each strain was then classified based on its spore structure and morphology according to Li et al., [17]. The 16S rDNA sequence (~1.5 kb) was sequenced by the Sanger method at DNA Technologies IDT (Singapore) based on amplicon amplified by PCR using primers p27F (5'-AGAGTTTGATCCTGGCTCAG-3') and p1492R (5'-GGTTACCTTGTTACGACTT-3') [18]. The DNA sequence was analyzed using Chromas version 2.4 and compared with published sequences in the GenBank database using BLAST software.

2.6. Investigating the Effects of Culture Medium, Temperature, Incubation Time and Plant Extract on the Antibacterial and Antifungal Activities of the Selected Actinomycetous Strains

Effect of culture medium: The selected strains were cultured on the tested media at

30 °C for 7 days and then tested for their antimicrobial activities. The tested media included ISP4, ISP4 with potato extract, YS, and Gauze No. 1.

Effect of temperature: The selected strains were cultured on the best medium (determined above) at the tested temperatures (20 °C, 30 °C, or 37 °C) for 7 days and then tested for their antimicrobial activities.

Effect of incubation time: The selected strains were cultured on the best medium (determined above) at the most suitable temperature (determined above) for 3, 4, 6, 7, 8, or 10 days, and then tested for their antimicrobial activities.

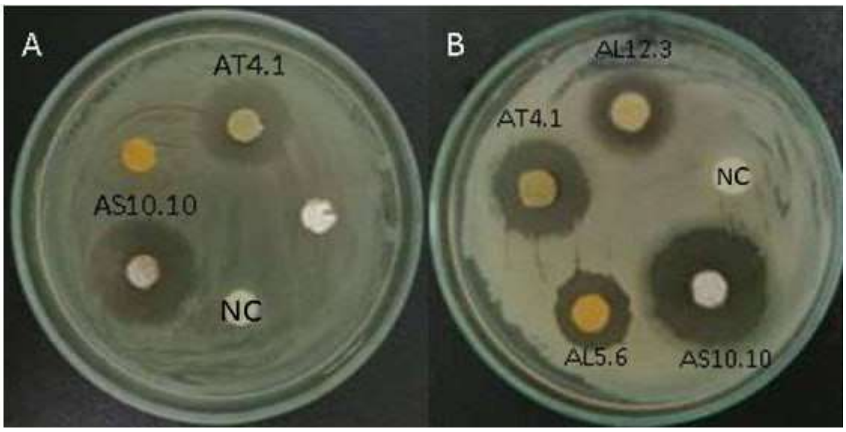
Effect of plant extract: Plant extracts (in ethanol or water) were prepared by mixing the biomass of each plant of interest (in powder) with ethanol or water at a ratio of 1:10 (w/v). After drying such an extracting solution in a rotary evaporator, the residual extract was obtained and dissolved in a 10% DMSO solution to obtain a stock solution having the final concentration of 50 mg extract/mL. The plant extract effect on the antimicrobial activities of the selected strains was evaluated by i) Adding the tested extract to the best culture medium (determined above) to the concentration of ~ 50 mg/L (by mixing the respective DMSO stock solution to the medium at the volumetric ratio of 1:1000); ii) Culturing the strains on such media at the most suitable temperature (determined above) in the most suitable incubation time (determined above), and then; and iii) Testing the cultures for their antimicrobial activities.

The ANOVA test was used to check whether the differences in the antimicrobial activities of the actinomycetous strains between different experimental cases were statistically significant or not [20].

3. Results and Dicussion

3.1. Screening and Identification of the Actinomycetous Strains with Good Antibacterial and Antifungal Activities

The studied actinomycetous strains were screened for their antibacterial activity against a representative Gram-positive bacterium (*B. subtilis*), a representative Gram-negative bacterium (*E. coli*), and three fungi (*C. albicans*, *A. flavus*, and *A. fumigatus*). The results of the screening (Table 2) showed that, out of the 136 studied strains, 5 strains were resistant to *E. coli*, 22 strains were resistant to *B. subtilis*, 3 strains were resistant to *C. albicans*, 3 strains were resistant to *A. flavus*, and 3 strains were resistant to *A. fumigatus* (Figure 1).



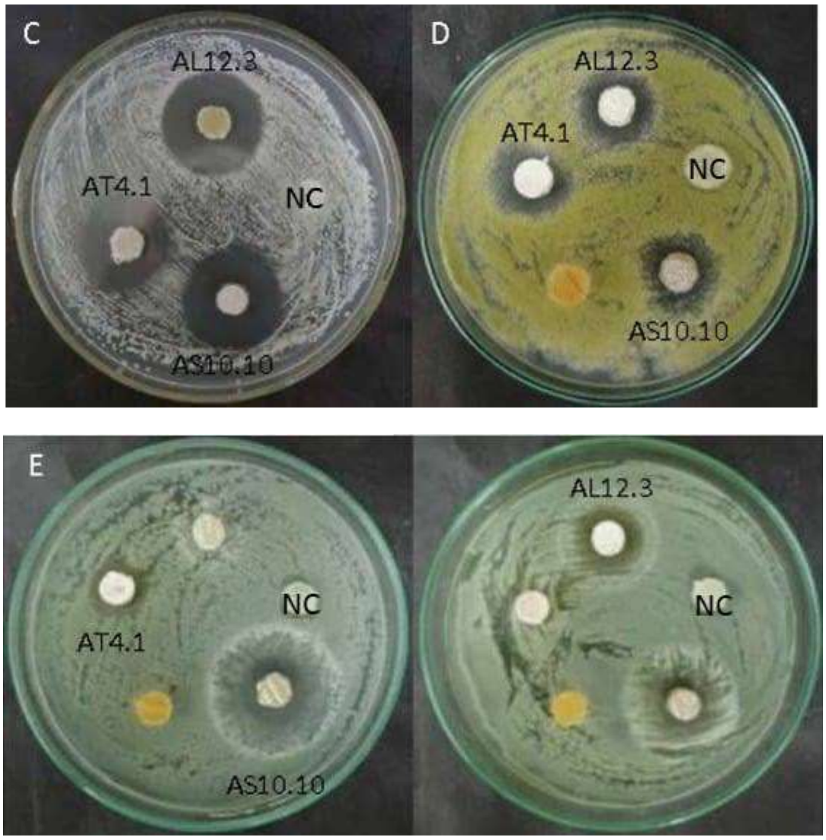


Figure 1. Agar diffusion plate assay showing the inhibitory activities of some strains against *E. coli* (A), *B. subtilis* (B), *C. albicans* (C), *A. flavus* (D) and *A. fumigatus* (E). NC: negative control.

Table 2. Summary of the antimicrobial activities of the actinomycetous strains displaying such activities

Strains	*E. coli*	*B. subtilis*	*C. albicans*	*A. fumigatus*	*A. flavus*
AT4.1	++	+++	++	+	+
AL5.6	-	++	-	-	-
AL12.3	-	+	++	+	+
AR17.4	-	++	-	-	-
AT20.1	-	++++	-	-	-
AT20.2	-	+++	-	-	-
AT20.5	-	++	-	-	-
AT20.6	-	++	-	-	-
AS4.2	-	++++	-	-	-

AS5.2	-	++++	-	-	-
AS6.4	-	++++	-	-	-
AS6.5	-	+++	-	-	-
AS7.1	-	++	-	-	-
AS7.2	-	+++	-	-	-
AS9.1	-	+	-	-	-
AS10.1	-	++	-	-	-
AS10.5	-	+++	-	-	-
AS10.8	+	+	-	-	-
AS10.10	+++	+++	++	+	++
AS11.4	+	+	-	-	-
AS12.7	-	+	-	-	-
AS13.8	+	+++	-	-	-

Note: bold text: endophytic actinomycetous strains; the rest: surface-associated actinomycetous strains; strong activity (+++): when the diameter (in mm) of the inhibition zone was > 12; relatively strong activity (++): when the diameter (in mm) of the inhibition zone was from 6-12; moderate activity (+): when the diameter (in mm) of the inhibition zone was < 6; no activity (-): when there was no inhibition ring.

By comparing the activity of the strains, we selected three actinomycetous strains, including two endophytes (AT4.1 and AL12.3) and one surface-associated one (AS10.10), which displayed broad-spectrum antimicrobial activities (Table 3), for further study.

It is interesting to note that those three selected strains were obtained from the plants that are known for their antimicrobial activities, including *Equisetum ramosissimum* (KT04), *Ficus semicordata* Buch-Ham.ex Sm (KT10), and *Tacca chantrieri* André (KT12). Specifically, KT04 is known to be effective in curing eye infections and dysentery, KT10 in treating bladder infections and leprosy, and KT12 in treating intestinal tract infections [22]. Thus, the results suggest that there might be certain links between the strains and the antimicrobial activities of the host plants.

We conducted the identification of the three selected actinomycetous strains by combining morphological observation with 16S rDNA sequencing. In terms of morphology, all three strains shared some characteristics, such as round, raised colonies, aerial hyphae that developed from white to brown, and straight spore chains. These characteristics suggest that they are *Streptomyces* spp. [21] (Figure 2). The 1.5 kb 16S rDNA fragments of the strains were sequenced and all the three sequences from the strains were identical to the corresponding ones of *Streptomyces* spp. with a 100% similarity. Therefore, based on the results of morphological observation and 16S rDNA sequencing, we concluded that the three actinomycetous strains are all *Streptomyces* spp.

Table 3. Antimicrobial and antifungal activites of the selected actinomycetous strains, demonstrated by the average diameters (in mm) of inhibition zones in agar diffusion assays

Test microorganism	AL12.3	AT4.1	AS10.10
E. coli	0	12.67±1.15	19±1
B. subtilis	3±1	15.67±1.54	19±1
C. albicans	12.67±0.57	10.67±2.3	13±1.73
A. flavus	3.33±0.57	2.33±0.57	2.67±0.57
A. fumigatus	3.33±0.57	1.67±0.57	8.33±0.57

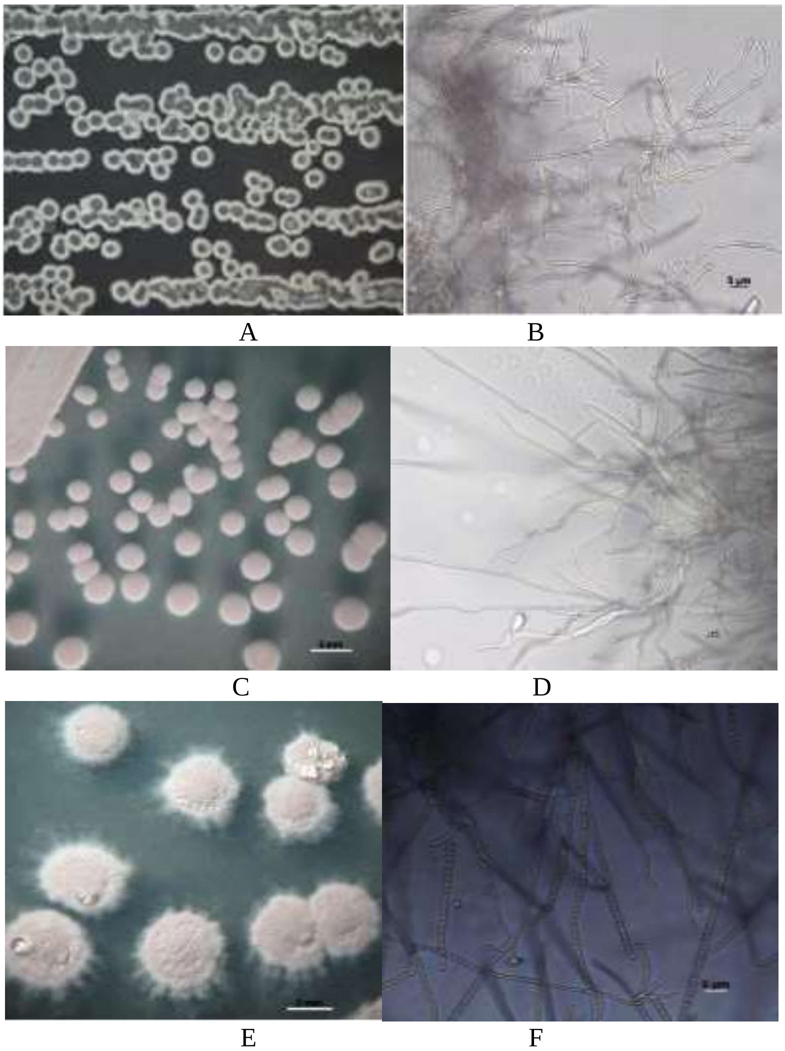


Figure 2. Characteristics of colonies and spores of strains AL12.3 (A, B), AT4.1 (C, D), and AS10.10 (E, F).

3.2. Effects of Culture Medium, Temperature, Incubation time and Plant Extract on the Antibacterial and Antifungal Activities of the Selected Actinomycetous Strains

The three selected strains were cultured on different media and their antibacterial and antifungal activities were tested. The results showed that the best activities were obtained when culturing the three endophytic and epiphytic actinomycetous strains on ISP4 medium supplemented with potato extract (Figure 3).

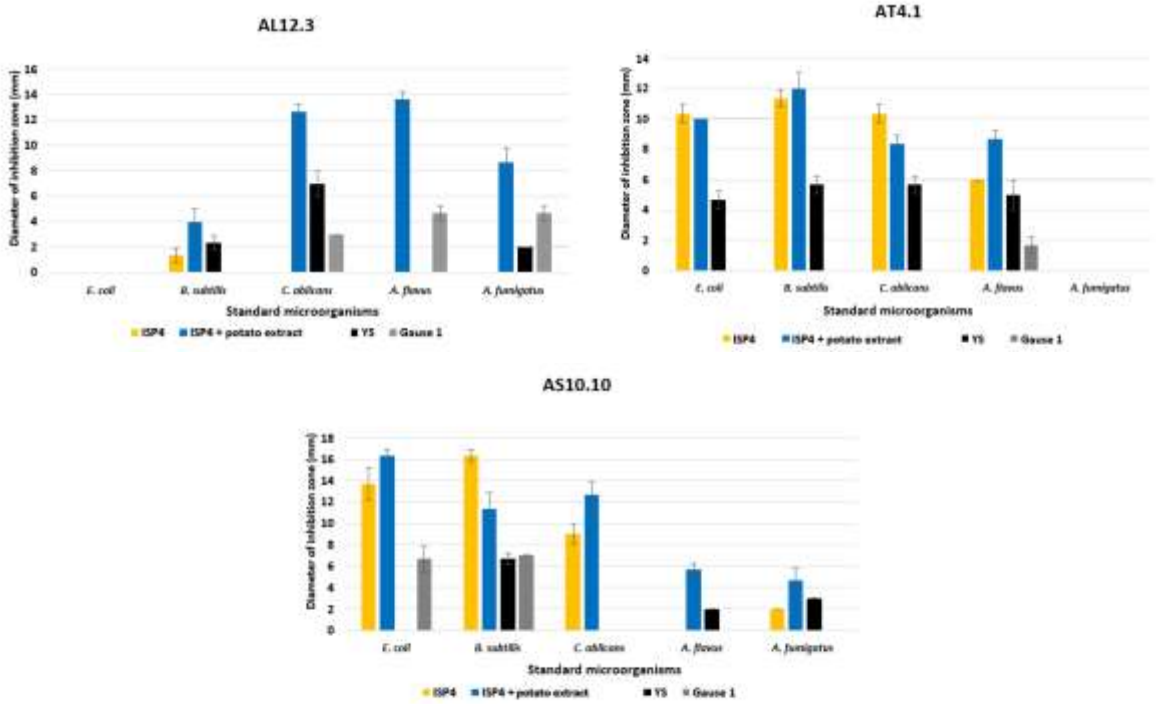


Figure 3. Effect of the culture medium on the antimicrobial activities of the selected actinomycetous strains.

The strains were cultured on ISP4 medium with added potato extract and incubated at different temperatures. The result showed that the best activities were obtained when the strains were cultured at a temperature of 30 °C (Figure 4).

To evaluate the effect of culture duration on the antimicrobial activities of the selected strains, they were cultured on ISP4 medium with potato extract, incubated at 30 °C, for different periods of time and then tested for their antimicrobial activities. For strain AL12.3, the antimicrobial activities against the tested microorganisms were the best after 6-7 days of culture (Figure 5). For strains AT4.1 and AS10.10, the antimicrobial activities against *E. coli*, *B. subtilis*, and *C. albicans* were good and similar to each other, regardless of the culture duration. The antimicrobial activity against *A. flavus* was the best after 6 days of culture for AT4.1 and after 3 days of culture for AS10.10, while that against *A. fumigatus* was the best after 3 days of culture for both strains (Figure 5).

After investigating the effects of environment, temperature, and culture duration on actinomycetous strains, we selected the most suitable conditions for the culture of the strains to obtain their best antimicrobial activities as follows: ISP4 medium with added potato extract, temperature at 30 °C, and culture duration of 7 days.

The next question is whether the activities of the strains can be changed when growing with materials from the repsective host plants. Therefore, we selected the most prominent strain, AT4.1, and tested its antimicrobial activities after culturing it on the media with the added extract of its respective host plant and on those without such material (the control) (Figure 6).

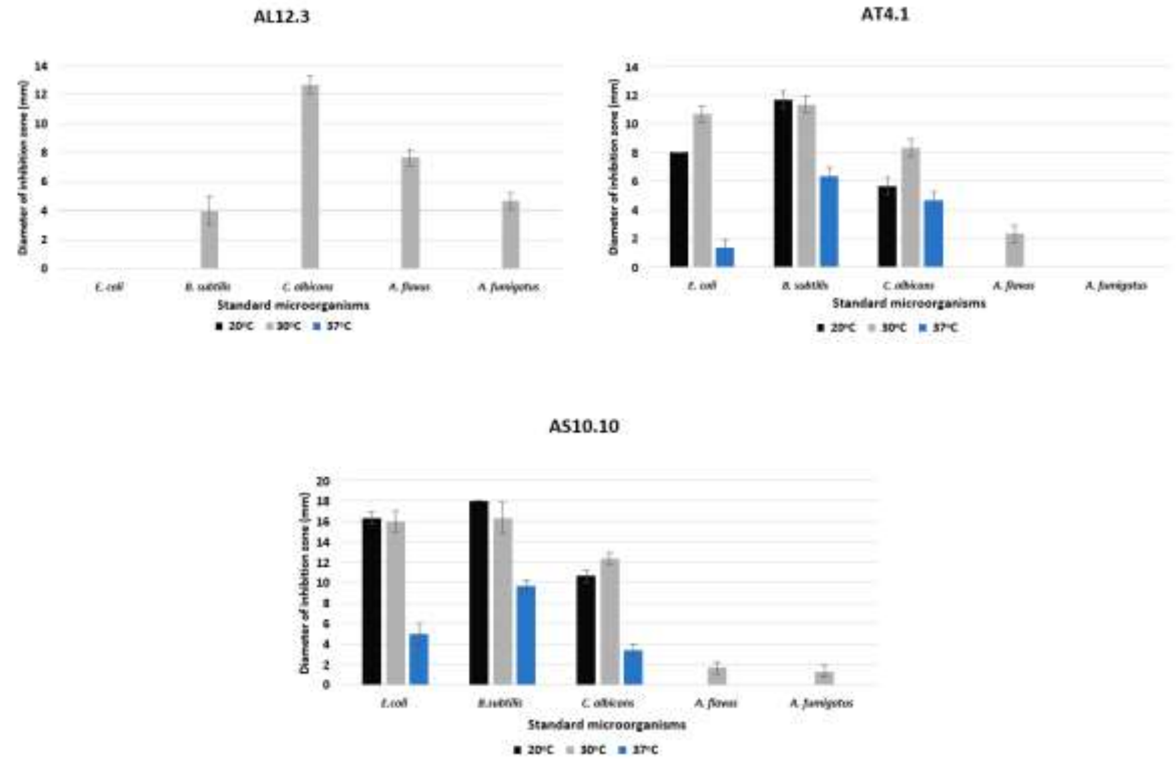
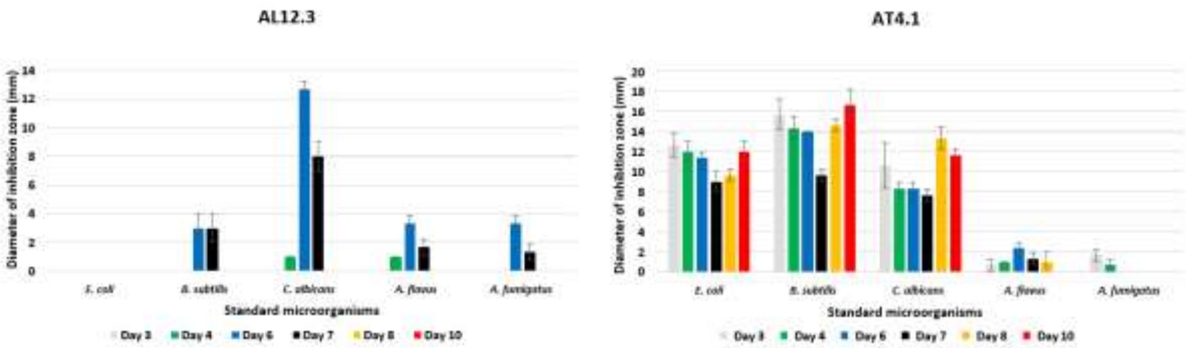


Figure 4. Effect of culture temperature on the antimicrobial activities of the selected strains.



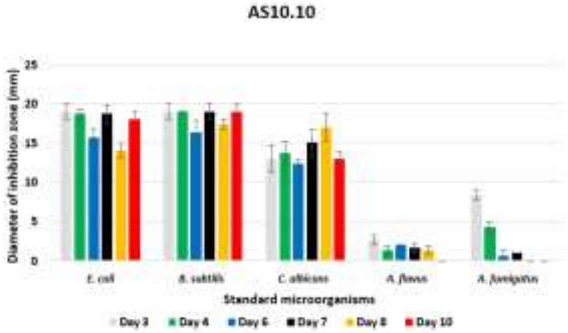


Figure 5. Effect of culture duration on the antimicrobial activities of the selected actinomycetous strains.

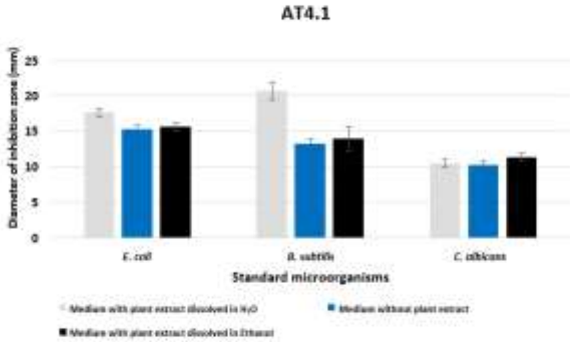


Figure 6. Effect of the host plant extract on the activity of strains AT4.1.

The results (Figure 6) showed that the plant extract in water increased the inhibitory activities of strain AT4.1 against *E. coli* and *B. subtilis* by about 15% and 30%, respectively, while the plant extract in ethanol only slightly increased the activity of strain AT4.1 against *B. subtilis* ($p < 0.05$). The effect of the extracts on the antifungal activity was not significant ($p > 0.05$). However, the results with the plant extracts in water are some convincing evidence of the enhanced biological activity of the actinomycetes when cultured with the host plant material, although the tests in this study are only preliminary.

This study highlights the importance of microorganisms associated with medicinal plants, by demonstrating that many of such microorganisms can display bioactivities (such as antibacterial and antifungal activities in this study) that may contribute to those of the plants. The fact that the prominent strains are *Streptomyces* spp. are in line with the findings of many previous studies [4, 6], suggesting that even in a characteristic environment in a plant body, *Streptomyces* species are still the majority among the bioactive actinomycetes. Indeed, in the previous study, *Streptomyces* species were the second most abundant group we obtained from the studied medicinal plants [14], which suggests their certain roles to the plants. The effect of the host plant extracts demonstrated in this study suggests that the host plants and the associated actinomycetes may have mutual interactions that enhance the bioactivities of both sides. Therefore, this study provides another foundation for further research on the interactions between endophytic microorganisms (and endophytic actinomycetes in particular) and plants.

4. Conclusion

In this study, we investigated the antibacterial and antifungal activities of 136 actinomycetous strains associated with 17 medicinal plants. Among the strains, three strains (AL12.3, AT4.1, and AS10.10) showed outstanding activities against the tested microorganisms. These three strains were identified as *Streptomyces* spp.. The most suitable culture conditions for the strains to produce the strongest activities are as follows: ISP4 medium with added potato extract, temperature of 30 °C, and culture duration of 6-7 days for AL12.3, and 3-4 days for AT4.1 and AS10.10. For strain AT4.1, the aqueous extract of its host plant increased its activity against both *E. coli* and *B. subtilis*, while the ethanol extract slightly increased its activity against *B. subtilis*. The results suggest that the roles of materials from host plants to the bioactivities of their associated microorganisms deserve more investigation.

Acknowlegements

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