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Diversity and antibacterial activity of *Phyllosticta* species

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ABSTRACT

***Phyllosticta* fungi are widely distributed and appear to have different lifestyles. Although some *Phyllosticta* species are known to cause plant diseases, others are useful due to their bioactive metabolites. In this study, we screened and isolated the *Phyllosticta* fungi from several plant specimens. In total, 67 *Phyllosticta* isolates were identified based on their distinct morphological characteristics. Of these, 18 isolates were pathogens and 49 isolates were endophytes. Besides, 61 isolates (91%) were identified as *P. capitalensis* indicating its widespread distribution. Thirty *Phyllosticta* isolates were then selected for studying their antibacterial activity. For this, the fungal strains were cultured in potato dextrose broth and cultivated at 27 C for 2 weeks. The fungal mycelia were removed and the culture supernatants were extracted using ethyl acetate. Antibacterial activity screening was then carried out using an agar disc diffusion assay. Our data showed that most *Phyllosticta* crude extracts (87%) were active and could inhibit at least one of the testing bacteria.**

Key words: Antibacterial activity, diversity, *Phyllosticta*, secondary metabolites.

INTRODUCTION

Phyllosticta species are globally widespread with a wide host range. Several species are important plant pathogens and responsible for numerous diseases (*i.e.*, leaf and fruit spots). For example, *P. citricarpa* (McAlpine) Aa, known to

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cause citrus black spot, is regarded as a quarantine pest[3]. *P. ampelicida* (Engelm.) Aa and *P. musarum* (Cooke) Aa cause the diseases in grapevines[8] and banana[11]. Other species have also been isolated as endophytes, such as *P. capitalensis* Henn.[10]. *Phyllosticta* endophytes are in particular of great interest. It is hypothesized that the fungal endophytes may help promoting plant health by providing resistance to hosts against environmental stresses[4]. Besides, many endophytes are highly regarded as an outstanding source of novel natural compounds that can be used in various applications[5]. Presently, *Phyllosticta* fungi are known to synthesize many active compounds[1,2]. These metabolites including phyllostictines, phyllostoxin, and phyllostin, exhibit interesting biological activity and their applications are remarkable ranging from biocontrol[2] and herbicide in agriculture[12] to antibiotics and anticancer agents in medicine[1]. Considering the importance of *Phyllosticta*, we therefore investigated their distribution in natural plant hosts of northern Thailand. Additionally, the antibacterial activity of *Phyllosticta* crude extracts was also studied.

MATERIALS AND METHODS

Screening and isolation of Phyllosticta *strains*. Diseased and healthy leaves from various host plants were collected in northern Thailand and used for screening *Phyllosticta* species. If pycnidia were present on diseased tissue, a single spore isolation was used to isolate the fungal cultures. To obtain isolates of *Phyllosticta* from diseased leaves when the pycnidia were not present, the leaf surface was cleaned by wiping with 70% ethanol. Small pieces of leaf were then cut from the interface between healthy and diseased tissue. These were surface sterilized in 70% ethanol, washed, and plated onto malt extract agar (MEA, per litre: 30 g malt extract, 5 g peptone, and 15 g agar). For isolation of endophytic fungi, explants (*i.e.*, leaf, bark, stem, bud) were washed in tap water and wiped with 70% ethanol. They were cut into small pieces (about 1 x 1 cm), suspended in 70% ethanol for 15 min (3 times) and washed in distilled water (3 times) before placing on MEA. All plates were incubated at 27 C for one week. The plates were generally observed daily or until the mycelial growth of the fungus could be seen. The hyphal tips of *Phyllosticta* colonies were then cut and transferred to fresh MEA plates. Cultural characteristics of the isolated fungi were assessed for identifying *Phyllosticta* species; these included distinct shape and structure of the fungal pycnidia and conidia[7]. The fungal isolates were deposited at the Mae Fah Luang University Culture Collection (MFLUCC).

Preparation of fungal extracts. Fungal strains were cultured in potato dextrose broth (PDB) at 27 C with shaking (130 rpm) for 2 weeks. Fungal mycelia were then removed by filtration using the filter paper Whatman no. 1. The culture supernatant was collected and mixed with 1X volume of ethyl acetate. The extraction was carried out twice. Crude extracts obtained were finally concentrated at room temperature for 48 h and were kept at -20 C until use.

Antibacterial activity assay. Fungal crude extracts were tested for antibacterial activity against four Gram-positive bacteria (*Bacillus cereus* TISTR687*, Bacillus subtilis* TISTR008*, Micrococcus luteus* TISTR884*, Staphylococcus aureus* TISTR1466), and three Gram-negative bacteria (*Escherichia*

coli TISTR780, *Pseudomonas aeruginosa* TISTR781, *Salmonella typhimurium* TISTR292). These bacterial strains were obtained from the TISTR (Thailand Institute of Scientific and Technological Research) Culture Collection. The antibacterial activity was undertaken using a disc diffusion assay. For this, a sterilized disc paper was immersed into fungal crude extracts for 48 h and placed on the inoculated plates. The diameter of the clear zones was then recorded.

RESULTS AND DISCUSSION

Phyllosticta species were isolated from healthy, symptomless leaves and diseased leaves of 45 plant hosts (**Table 1**). The fungal species were initially screened based on their distinct characteristics (*i.e.*, pycnidia, conidiomata, conidia, and cultural characteristics) [**Fig. 1**]. In total, 67 *Phyllosticta* isolates were obtained and, mainly based on morphology, further characterized as *P. capitalensis* Henn. (61), *P. cordylinophila* P.A. Young (3), *P. citrimaxima* S. Wikee, Crous, K.D. Hyde & Mckenzie (1), and *P. mangiferae* Bat. (2) [**Table 1**]. *P. capitalensis* appears to be a common species. This species is widely found in a broad range of hosts including ornamental plants, woody plants, fruits, and vegetables. Besides, three *Phyllosticta* isolates, identified as *P. capitalensis*, were firstly screened from *Brassica juncea* (WK080), *Canna indica* (WK077), and *Loranthaceae* (WK067) in Thailand. It should be noted, however, that the morphology of *Phyllosticta* species is similar. Further analysis using molecular approach should be carried out to confirm the identity of these *Phyllosticta* species. *P. capitalensis* in particular is suspected to be cryptic based on the multigene analysis[10]. It has also been reported that the sole use of the ITS region is insufficient for separating the species level of *Phyllosticta*[6]. Therefore, a phylogenetic study of *Phyllosticta* species is necessary and this is expected to shed light on its taxonomy.

Based on the isolating method, *Phyllosticta* species obtained in this study can be classified as pathogens and endophytes. *Phyllosticta* pathogens could be easily isolated from the diseased plant samples using single spore isolation technique. Our findings showed 18 *Phyllosticta* pathogens that were involved with the disease symptom of the plant hosts including *Mangifera* sp., *Musa* sp., and *Orchidaceae* (**Table 1**). *Phyllosticta* species are one of the most important fungal pathogens causing a serious disease in several plant hosts (*i.e.*, freckle disease of banana and tan spot on citrus). In Thailand, the situation is not serious; however, the occurrence of *Phyllosticta* on agricultural products may cause a problem during a quarantine inspection[3].

In addition, endophytic *Phyllosticta* species were obtained using the surface sterilization technique. This endophytic life mode is interesting as several studies showed that the fungal endophytes could produce many useful bioactive compounds[1,2]. *Phyllosticta* species in particular are of great interest as previous studies showed that many active compounds exhibiting interesting biological activities were derived from this fungal species[2,12]. In this study, a total of 49 *Phyllosticta* endophytes were recovered from various healthy plant hosts. These fungal endophytes were subjected to further study regarding their antibacterial activity.

For the antibacterial activity assay, 30 *Phyllosticta* endophytes were randomly

Table 1. List of *Phyllosticta* isolates obtained in this study and their plant hosts.

Original code	Species	Life mode[a]	Host[b]
WK002	*P. capitalensis*	P	Unknown
WK004	*P. capitalensis*	P	*Punica granatum* L.
WK005	*P. capitalensis*	P	*Schefflera venulosa* (Wight & Arn.) Harms
WK006	*P. capitalensis*	E	*Saccharum* spp.
WK007	*P. capitalensis*	P	*Arecaceae*
WK010	*P. capitalensis*	P	*Liliaceae*
WK011	*P. capitalensis*	P	*Ficus benjamina* L.
WK012	*P. capitalensis*	P	*Liliaceae*
WK013	*P. capitalensis*	P	*Orchidaceae*
WK016	*P. capitalensis*	P	*Dracaena sanderiana* Sander
WK017	*P. capitalensis*	P	*Liliaceae*
WK018	*P. capitalensis*	P	*Orchidaceae*
WK020	*P. capitalensis*	P	*Cordyline fruticosa* (L.) A. Chev.
WK021	*P. capitalensis*	P	*Arecaceae*
WK022	*P. capitalensis*	P	Unknown
WK023	*P. capitalensis*	P	*Liliaceae*
WK024	*P. capitalensis*	P	*Cordyline fruticosa* (L.) A. Chev.
WK026	*P. capitalensis*	P	*Liliaceae*
WK031	*P. capitalensis*	E	*Magnoliaceae*
WK032	*P. capitalensis*	E	*Mangifera indica* L.
WK033	*P. capitalensis*	E	*Euphorbiaceae*
WK035	*P. capitalensis*	E	*Polyscias* spp.
WK036	*P. capitalensis*	E	*Baccaurea ramiflora* Lour.
WK037	*P. capitalensis*	E	*Hibiscus syriacus* L.
WK038	*P. capitalensis*	P	*Ophiopogon japonicus* (Thunb.) Ker Gawl.
WK039	*P. capitalensis*	E	*Tectona grandis* L.f.
WK040	*P. capitalensis*	E	*Crinum asiaticum* L.
WK041	*P. capitalensis*	E	*Orchidaceae*
WK042	*P. capitalensis*	E	*Ixora chinensis* Lam.
WK043	*P. cordylinophila*	E	*Cordyline fruticosa* (L.) A. Chev.
WK044	*P. mangiferae*	E	*Mangifera* sp.
WK045	*P. capitalensis*	E	*Polyalthia longifolia* (Sonn.) Thwaites
WK046	*P. citrimaxima*	E	*Citrus maxima* (Burm. f.) Osbeck
WK047	*P. capitalensis*	E	*Pyrrosia adnascens* (Sw.) Ching
WK048	*P. cordylinophila*	E	*Cordyline fruticosa* (L.) A. Chev.
WK049	*P. capitalensis*	E	*Euphorbiaceae*
WK050	*P. capitalensis*	E	*Philodendron* sp.
WK051	*P. capitalensis*	E	*Piper nigrum* L.
WK052	*P. capitalensis*	E	*Phalaenopsis* spp.

Table 1 continued

WK053	*P. capitalensis*	E	*Swietenia macrophylla* G. King
WK054	*P. mangiferae*	E	*Mangifera* sp.
WK055	*P. capitalensis*	E	*Jasminum sambac* (L.) Aiton
WK056	*P. capitalensis*	E	*Nephelium lappaceum* L.
WK057	*P. capitalensis*	E	*Tectona grandis* L.f.
WK058	*P. capitalensis*	E	*Tectona grandis* L.f.
WK059	*P. capitalensis*	E	*Tectona grandis* L.f.
WK060	*P. capitalensis*	E	*Tectona grandis* L.f.
WK061	*P. capitalensis*	E	*Curcuma parviflora* Wall.
WK062	*P. capitalensis*	E	*Caryota mitis* Lour.
WK063	*P. capitalensis*	E	*Cordyline* sp.
WK064	*P. capitalensis*	E	*Calathea majestica* (Linden) H. Kenn.
WK065	*P. capitalensis*	E	*Orchidaceae*
WK066	*P. capitalensis*	E	*Elaeocarpus hygrophilus* Kurz
WK067	*P. capitalensis*	E	*Loranthaceae*
WK068	*P. capitalensis*	E	*Musa* sp.
WK069	*P. capitalensis*	E	*Baccaurea ramiflora* Lour.
WK070	*P. capitalensis*	E	*Caryota mitis* Lour.
WK071	*P. capitalensis*	E	*Piper retrofractum* Vahl
WK072	*P. capitalensis*	E	*Artocarpus heterophyllus* Lam.
WK073	*P. capitalensis*	E	*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.
WK074	*P. capitalensis*	E	*Dracaena cochinchinensis* (Lour.) S. C. Chen
WK075	*P. capitalensis*	E	*Duranta erecta* L.
WK076	*P. capitalensis*	E	*Dimocarpus longan* Lour.
WK077	*P. capitalensis*	E	*Canna indica* L.
WK078	*P. capitalensis*	E	*Ascocentrum miniatum* (Lindl.) Schltr.
WK079	*P. cordylinophila*	E	*Cordyline fruticosa* (L.) A. Chev.
WK080	*P. capitalensis*	E	*Brassica juncea* (L.) Czern.

[a] P: Pathogen. E: Endophyte.
[b] Plant hosts are given as taxonomic family or species.

selected and their crude extracts were prepared using ethyl acetate. The antibacterial activity of these crude extracts was then undertaken using the disc diffusion method. Based on this, our data revealed that most *Phyllosticta* crude extracts could inhibit the growth of at least one of the testing bacteria (**Table 2**).

Fig. 2 shows an inhibitory effect of some representative *Phyllosticta* crude extracts when assayed against the testing bacteria. Discs with a marked clear zone indicated an inhibitory activity of the fungal extracts, whereas those without clear zone suggested no activity including a control disc at the center of the plates.

It should be noted that interestingly the crude extracts of *Phyllosticta* strains WK052, WK057, WK061, WK063, and WK066 exhibited strong antibacterial activity against the tested bacteria as indicated by a wide clear zone ranging

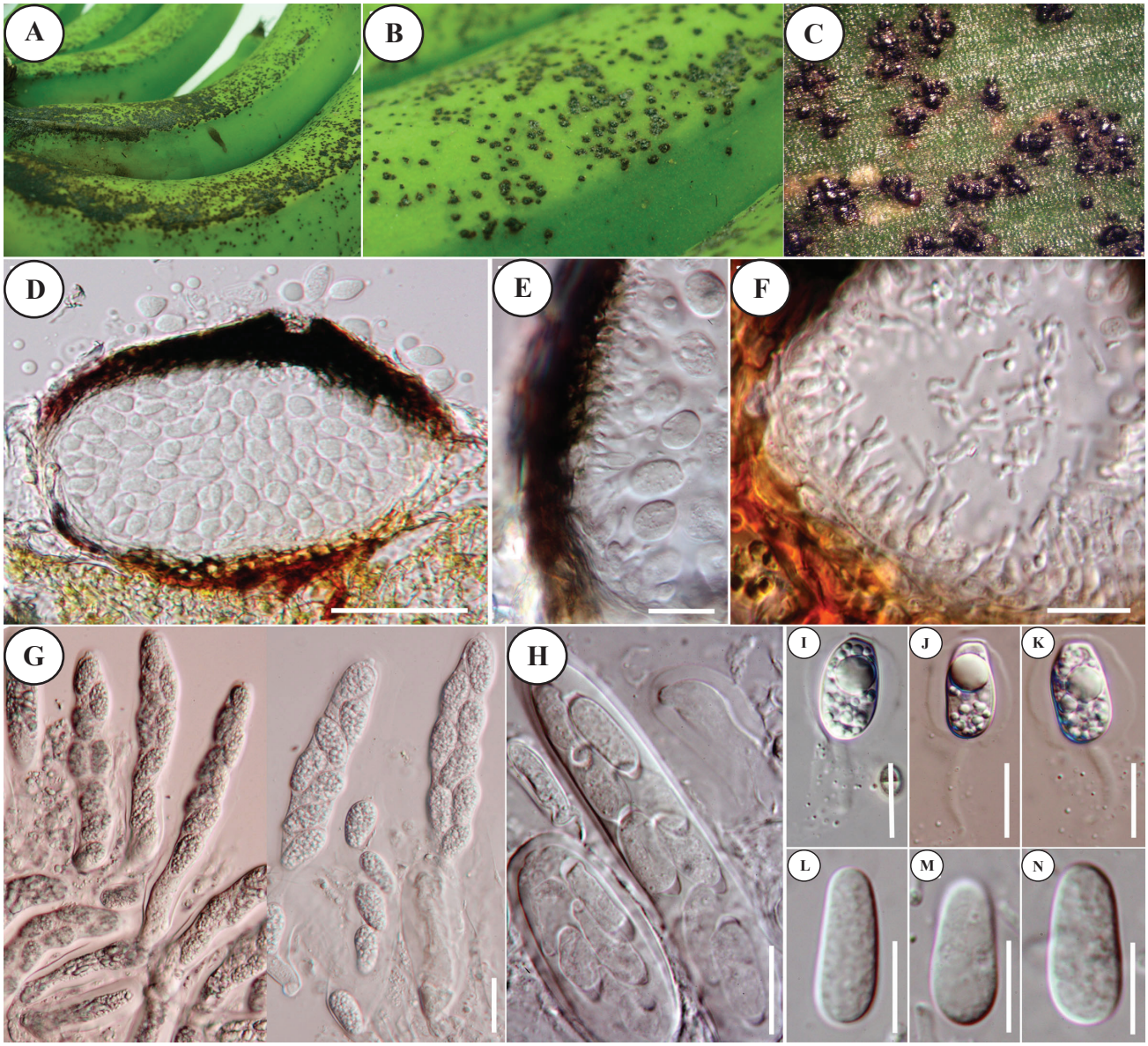

Fig. 1. Distinct characteristics of *Phyllosticta* fungus. *P. capitalensis* WK068 isolated from *Musa* sp. is given as an example. A-C: Diseased symptom present when infected. D-F: Pycnidia. G-H: Asci. I-N: Ascospore and conidia. Scale bar: D= 50 µm, E= 10 µm, F= 20 µm, G–N= 10 µm.

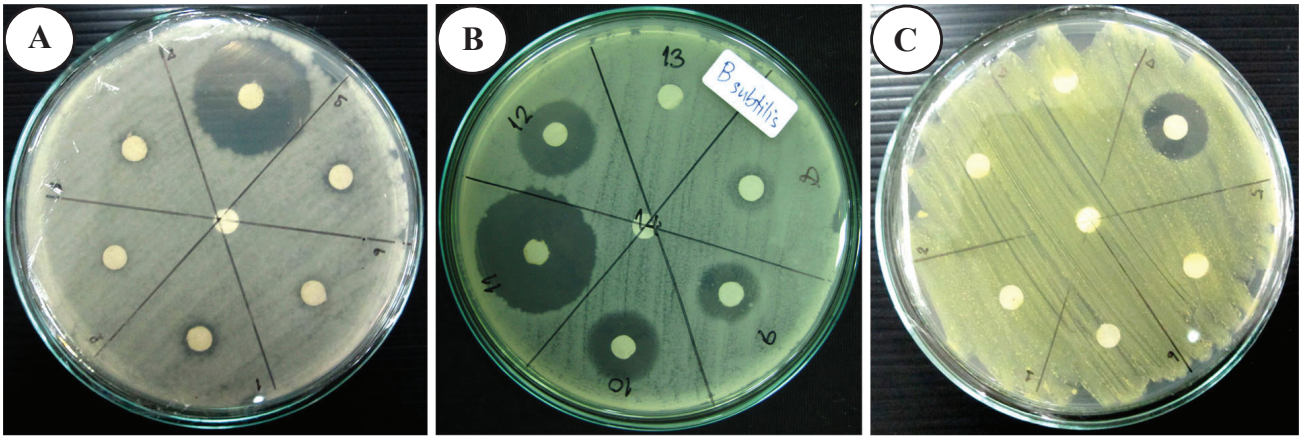

Fig. 2. Representatives of inhibitory activity of the crude extracts obtained from different isolates of *Phyllosticta* fungi. A: *P. capitalensis* WK066 on *Bacillus cereus*. B: *P. capitalensis* WK075, WK076, WK077, WK078, and *P. cordylinophila* WK079 on *Bacillus subtilis* (clockwise direction). C: *P. capitalensis* WK066 on *Micrococcus luteus*.

from 20 to 29 mm (**Table 2**). There were only 4 strains (WK051, WK065, WK071, WK072) that did not show any inhibitory effect when tested (**Table 2**). Moreover, the *Phyllosticta* crude extracts seemed to be more effective when testing against the Gram-positive bacteria (**Table 2**). This result is probably caused by a difference in bacterial cell wall, in which the *Phyllosticta* crude extracts affected the cell wall component and/or cell wall synthesis process of the Gram-positive bacteria. *Phyllosticta* species are known to produce various kinds of secondary metabolites[9]. Additionally, some *Phyllosticta* species have been evaluated for using as a biocontrol agent[12]. Our study is one of the few studies dealing with the antibacterial activity of *Phyllosticta* species. The present data confirmed a potential use of *Phyllosticta* species in terms of their antibacterial activity.

In conclusion, 67 *Phyllosticta* strains were isolated from a wide range of host plants. They were recovered as pathogens (18 isolates) and endophytes (49 isolates). *P. capitalensis* was the predominant species isolated in this study. Further investigation will be performed using a molecular approach. Most *Phyllosticta* crude extracts were active and inhibited the bacterial growth. Their antibacterial activity was interesting, and it deserves further exploration to study the *Phyllosticta* metabolites. The potential use of these *Phyllosticta* active compounds in medicine and biotechnology is encouraging.

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Table 2. Antibacterial activity of *Phyllosticta* species against Gram positive and Gram negative bacteria using the disc diffusion method. The data given are mean ± SD of the diameter of clear zones.

Phyllosticta	Inhibition zone (mm)						
code	BC	SA	ML	BS	EC	PA	ST
WK051	-	-	-	-	-	-	-
WK052	27.3±0.6	17.0±3.5	18.3±0.6	28.3±1.5	8.0±1.0	-	8.0±1.0
WK053	-	-	6.7±0.6	-	-	-	-
WK054	6.7±1.5	7.0±1.0	6.7±1.2	6.7±0.6	-	-	-
WK055	10.3±2.3	7.0±0.0	-	10.3±0.6	-	-	6.7±0.6
WK056	6.3±0.6	-	6.3±0.6	-	-	-	-
WK057	27.0±0	12±0.0	17.7±2.5	27.7±2.1	6.3±0.6	-	7.3±0.6
WK058	20.7±1.5	16.3±0.6	14.7±0.6	16.3±1.5	6.7±0.6	-	-
WK059	20.7±2.3	8.0±1.0	10.7±1.2	24.3±3.2	6.3±0.6	-	-
WK060	20.7±1.0	8.0±1.0	10.0±1.2	24.3±3.2	6.3±0.6	-	-
WK061	8.7±1.5	8.0±1.0	6.7±1.2	8.7±0.6	-	7.3±1.5	6.7±0.6
WK062	9.3±0.6	8.0±1.1	-	7.7±0.6	-	8.0±1.0	6.3±0.6
WK063	8.0±1.0	6.3±0.6	-	8.3±0.6	-	7.0±0.0	6.3±0.6
WK064	9.0±1.7	-	6.7±0.6	16.7±1.5	-	-	-
WK065	-	-	-	-	-	-	-
WK066	29±1.7	15.3±2.5	16.0±2.6	24.3±1.2	8.7±1.2	7.7±1.5	7.0±0.0
WK067	9.7±2.1	-	-	9.3±0.6	-	-	-
WK068	9.3±0.6	-	6.7±0.6	12.7±0.6	-	-	-
WK069	13.3±2.1	-	7.3±0.6	16.7±1.2	-	-	-
WK070	13.0±2.0	-	8.0±0.6	18.0±0.0	-	-	-
WK071	-	-	-	-	-	-	-
WK072	-	-	-	-	-	-	-
WK073	7.0±0.0	-	-	13.3±0.6	-	-	-
WK074	9.3±2.3	-	6.7±0.6	15.7±0.6	-	-	-
WK075	7.0±0.0	-	-	10.3±2.1	-	-	-
WK076	8.3±1.5	-	-	14.7±0.6	-	-	-
WK077	10.7±0.6	-	6.7±0.6	17.0±1.7	-	-	-
WK078	19.7±1.5	-	16.7±2.9	26.3±1.5	-	-	-
WK079	14.3±0.6	-	8.7±1.2	18.0±0.0	-	-	-
WK080	-	-	-	6.7±1.2	-	-	-

BC= *Bacillus cereus*. SA= *Staphylococcus aureus*. ML= *Micrococcus luteus*. BS= *Bacillus subtilis*. EC= *Escherichia coli*. PA= *Pseudomonas aeruginosa*. ST= *Salmonella typhimurium*.
- = No inhibition zone.

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