

Biological Activities of Endophytic *Xylaria* sp. Isolated from Tropical forest in Chaipayoom Province, Thailand

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Abstract:

The ultimate goal in this study is to screen biological activities from endophytic *Xylaria* sp. isolated from leaf of 4 Dipterocarp forest tree species such as *Cinnamomum iners*, *Shorea siamensis*, *Fernandoa adenophylla*, *Quercus semiserrata*. All endophytic fungi isolates were obtained and identified based on morphological characteristics. Sixty-four from 125 endophyte isolates were belong to *Xylaria* genera. Moreover, the typical endophytic fungi genera such as *Phomopsis* spp., *Pestalotiopsis* sp., *Colletotrichum* sp., *Phyllosticta* sp., *Daldinia* sp., *Aspergillus* sp., *Mycelia sterilia* spp., were commonly found in tropical forest. *Xylaria* isolate sp.1 was the only one isolate that showed excellent broad spectrum antimicrobial against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*. Moreover, *Xylaria* isolate sp.9 showed strong inhibitory activity against all test bacteria. Moreover, *Xylaria* sp.1 showed the best efficiency of cytotoxicity against Jurkat cell line with IC₅₀ value of 2.63 µg/mL and *Xylaria* sp.2 showed best efficiency of cytotoxicity against HEK293 cell line with IC₅₀ value of 2.94 µg/mL.

Key words: Endophytic fungi, *Xylaria* sp., Antimicrobial activity, Anticancer activity, Bioactive compound

1. Introduction

In recent years, drug resistance in pathogens and the occurrence of cancer in the world's population has become a serious medical problem. Therefore, the needs for useful novel bioactive compounds to assist and relieve all aspects of the human conditions are required. Metabolites from microorganism have been considered to determine for the new potential source. The fungi, especially endophytic fungi are the best one that shows high potential as sources of novel antiviral, anticancer, antioxidant, and insecticidal compounds. Endophytes are organisms that live inside plant tissues for at least part of their life cycle. However, they are totally harmless to the host [1]. They produced potential bioactive compounds, and sources of novel metabolites for medicine. Several in the group of bioactive compounds derived from endophytic fungi including compound, alkaloid, steroids, terpenoid, phenols, quinines and flavonoids, exhibited several biological activities such as anti-cancer [2], anti-fungal [3], anti-oxidant [4], anti-microbial [5], anti-inflammatory [6] and anti-viral activities [7].

One of the most interesting endophytic fungi is in the Family of Xylariaceae. The Xylariaceae are the largest and relatively well-known fungal family in phylum Ascomycota. It has representatives especially in the tropics and subtropics. The fungi in this group such as *Daldinia*, *Hypoxylon*, *Xylaria* which are wood-decay characters that are able to break down the major components of wood.

Xylaria spp. are a wood-decay fungi, commonly found in tropical region. Sometime in their life cycle, fungi in genus *Xylaria* are usually found as both saprophyte and endophyte. Moreover, it has been well reported as the rich source of bioactive compounds such as terpenoids, xyloketal, xanthenes and cytochalasins [8-11]. They are very useful in biological activities for example anticancer, antifungal, antioxidant, antimicrobial, anti-inflammatory and antiviral.

Thailand is situated in a tropical region and is considered one of the areas with greatest diversity of Xylariaceae [12]. Interesting metabolites isolated from *Xylaria* sp. can be expected. A proposal of this study is to isolate endophytic *Xylaria* sp. from Phukhiew Wildlife Sanctuary, Chaiyaphum Province, Thailand. The isolated endophytes identification are based on morphological methods and the screening of their metabolites were done by antimicrobial assay and cytotoxic activity.

2. Materials and Methods

A. Site of plants collection

Healthy plant leaves which are *Cinnamomum iners*, *Shorea siamensis*, *Fernandoa adenophylla*, *Quercus semiserrata* were collected from Phukhiew Wildlife Sanctuary at Chaiyaphum Province, Thailand. All samples were stored in polythene bags and maintained in an icebox. After that, they were used to isolate fungal endophyte within 48 h after collection.

B. Isolation and identification of endophytic fungi

Healthy plant leaves were carefully cleaned with running tap water. Then, all samples were cut into small pieces of approximately 5×5 mm². The samples were surface sterilized using the method described by Blodett [13]; the samples were immersed in 95% ethanol for 1 min, 10% sodium hypochlorite solution for 5 min and 95%

ethanol for 30 seconds and then rinsed in sterile water. The surface sterile samples were placed on Potato Dextrose Agar (PDA) and incubated at 30 °C for 7 days. The hyphal tips of the endophytic fungus growing out from the plant tissue were subsequently transferred to fresh PDA plate and incubated at room temperature for 7-14 days. The cultures purity were identified under a light microscope and identification to the genus level was undertaken by referring to the key described by Barnett and Hunter (1987) and Von Arx (1978) [14, 15].

C. Fermentation and extraction of endophytic *Xylaria* spp.

Cultures of the *Xylaria* spp. on PDA were transferred into Malt Extract Broth (MEB) in Erlenmeyer flasks 250 ml, under static condition and incubated for 6 weeks at room temperature (25-30 °C). The fermentation broth was filtered. Then, all filtrated culture were extracted three times with Ethyl Acetate (EtOAc). Each extracted solution was concentrated in a rotary evaporator. Dried crude extracts were prepared for examination of their biological activities by dissolving in dimethyl sulphoxide (DMSO).

D. Antimicrobial activity

In this study, pathogenic microorganisms including, two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633) and two Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739) and the yeast, *Candida albicans* (ATCC 90028) were used to determined antimicrobial activity by paper disc diffusion method [16]. From this procedure, cotton sticks were dipped in inoculated suspension of tested microorganisms. Then, impregnated cotton sticks were streaked on dry Nutrient Agar (NA) by for bacteria and Sabouraud Dextrose Agar (SDA) for yeast. The discs (6 mm in diameter) saturated with crude extracts solutions were placed on the inoculated agar. The diameters of inhibition clear zones were measured for the antimicrobial activity. Streptomycin and Nystatin were used as positive controls for bacteria and yeast, respectively.

E. Cytotoxic activity

Human T lymphocyte cells (Jurkat) and Human Embryonic Kidney 293 cell (HEK293) cell line (1×10^5 cells/ml in 100 μ l culture) were treated with the crude extract at various concentrations and incubated at 37 °C in a humidified atmosphere with 5% CO₂ for 4 days in RPMI 1640 medium with 10% Fetal Bovine Serum (FBS). Cytotoxic activity was estimated by using MTT (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrasodium bromide) assay. At the end of treatment, 0.4 N HCl in isopropanol was used to dissolve formazan and the absorbance was measured at 540 nm. Percentages of cell viability were calculated as ratio of the A₅₄₀ values of treated and control cells (treated with 1% DMSO) The efficacy of crude extract for their cytotoxic activity against cancer cell line was determined by Inhibition Concentration (IC₅₀) (Olechno *et al.*, 2006) [17]

$$\text{Percentage of cell viability (\%)} = \frac{(\text{OD test} - \text{OD blank}) * 100}{\text{OD control} - \text{OD blank}}$$

3. Results and Discussion

Healthy leaves of the 4 plants collected from Phukhieo Wildlife Sanctuary at Chaiyaphum Province, Thailand, were collected for isolation of endophytic fungi by sterile technique. A total of 125 isolates were isolated, including 64 *Xylaria* spp., and non-*Xylaria* sp. including 9 *Phomopsis* spp., 7 *Pestalotiopsis* sp., 4 *Colletotrichum* sp., 3 *Phyllosticta* sp., 12 *Daldinia* sp., 8 *Aspergillus* sp., and 18 *Mycelia sterilia* spp., respectively. (Fig.1). A list of isolates is shown in Table 1. All isolates were maintained on potato dextrose agar

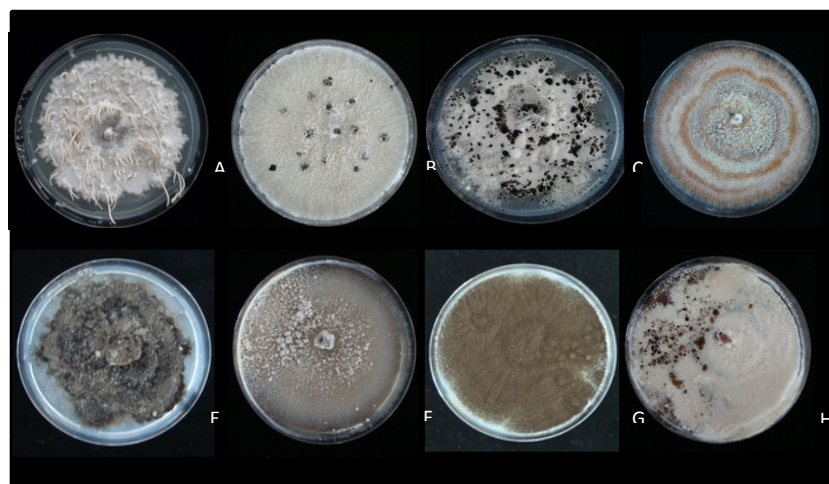


Figure 1. Endophytic fungi colony on Potato Dextrose Agar after incubated 14 days. A *Xylaria* sp., B *Phomopsis* sp., C *Pestalotiopsis* sp., D *Colletotrichum* sp., E *Phyllosticta* sp., F *Daldinia* sp., G *Aspergillus* sp., H *Mycelia sterilia*

Table 1. Numbers of endophytic fungi isolated from plant leaves

Endophytic fungi	Host species				Isolates
	CI	SS	FA	QS	
<i>Xylaria</i> spp.	17	23	4	20	64
<i>Phomopsis</i> spp.	-	3	3	3	9
<i>Pestalotiopsis</i> sp.	5	-	2	-	7
<i>Colletotrichum</i> sp.	-	-	4	-	4
<i>Phyllosticta</i> sp.	-	-	3	-	3
<i>Daldinia</i> sp.	-	5	7	-	12
<i>Aspergillus</i> sp.	3	-	5	-	8
<i>Mycelia sterilia</i> spp.	8	1	-	9	18
Total	33	32	28	32	125

Refer to Table 1 for hosts code **CI** = *Cinnamomum iners*, **SS** = *Shorea siamensis*, **FA** = *Fernandoa adenophylla*, **QS** = *Quercus semiserrata*

Fungal hyphal tips that germinated from Leaf segment were carefully observed for important characteristic mycelium of genus *Xylaria*. The mycelia were assumed by linear, non-dendritic branching, silky, and white hyphae at the initial growth (Fig.2A) [18]. Stroma structures, typical of fungi in genus *Xylaria*, were obtained after 4 weeks. (Fig.2B)



Figure 2. Colony character of *Xylaria* sp. Initially white colony on PDA (A) Stromata structure on Potato Dextrose Agar (B)

Endophytic *Xylaria* species are also found in other places of Thailand. Endophytic *Xylaria* sp. PSU-D14 isolated from the leaves of *Garcinia dulcis* in Songkhla Province, showed antifungal activity against *Candida albicans* [19]. Moreover, *Xylaria* species were found in the northern part of Thailand such as Doi Suthep National Park in Chiang Mai, Naresuan University, in Phayao province [20], Deciduous Dipterocarp Forest forest in Nan (21) and Tak province (22). All of the 12 genera of *Xylaria* sp. were selected for screen antimicrobial activities. Crude extracts derived from extraction with ethyl acetate were adjusted concentration to 1 mg/ml for test activity against *Staphylococcus aureus* (ATCC 6538-P), *Bacillus subtilis* (ATCC 6633) and two Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739) and the yeast, *Candida albicans* (ATCC 90028). Streptomycin and Nystatin were used as positive control for bacterial test and yeast test, respectively. Antimicrobial activity result was shown in Table 2.

Sizes of inhibition zones were different among *Xylaria* sp. strain. The crude extracts of *Xylaria* sp.1 showed activity against all test microorganisms. *Xylaria* sp.9 inhibited the growth of all bacterial strain and showed highest inhibition activity against *Staphylococcus aureus* of about 11.5 mm. that closely antimicrobial activity of positive control. Moreover, Crude extracts of genus *Xylaria* inhibited the growth of Gram positive bacteria better than Gram negative bacteria and yeast. These differences may be attributed to the fact that the cell wall in the Gram-positive bacteria is a single layer, whereas the Gram-negative cell wall is a multi-layered structure and the yeast cell wall is quite complex [23].

Table 2. Effect of *Xylaria* sp. isolates crude extracts against the test microorganisms

<i>Xylaria</i> Isolates.	Zone of inhibition diameter (mm)				
	BAC	STA	ESC	PSE	CAN
<i>Xylaria</i> sp.1	7	9.75	9.5	9.5	8
<i>Xylaria</i> sp.2	-	10.5	-	10.5	-
<i>Xylaria</i> sp.3	-	-	-	-	-
<i>Xylaria</i> sp.4	-	-	-	-	-
<i>Xylaria</i> sp.5	-	-	-	-	-
<i>Xylaria</i> sp.6	-	6.6	-	7	-
<i>Xylaria</i> sp.7	9	-	-	-	-
<i>Xylaria</i> sp.8	9	8	-	8	-
<i>Xylaria</i> sp.9	10	11.5	8.5	8.75	-
<i>Xylaria</i> sp.10	-	-	-	-	-
<i>Xylaria</i> sp.11	-	-	-	-	-
<i>Xylaria</i> sp.12	-	-	-	-	-
Streptomycin	15	16	15	12	-
Nystatin	-	-	-	-	13

Refer to Table 2 for pathogenic microorganism test code **BAC** = *Bacillus subtilis*, **STA** = *Staphylococcus aureus*, **ESC** = *Escherichia coli*, **PSE** = *Pseudomonas auruginosa*, **CAN** = *Candida albicans*

Twelve of the crude extracts were tested for cytotoxicity against Jurkat and HEK293 cell lines *in vitro* by using the MTT methods. As indicated in Table 3, *Xylaria* sp.1 showed the highest efficiency of cytotoxicity against Jurkat cell line with IC₅₀ value of 2.63 µg/mL and *Xylaria* sp.2 showed the highest efficiency of cytotoxicity against HEK293 cell line with IC₅₀ value of 2.94 µg/mL, when cell line treated with DMSO was used as positive control and untreated cell was used as negative control. Both crude extracts from *Xylaria* sp.1 and *Xylaria* sp.2 were isolated from *Cinnamomum iners*.

Table 3 Growth inhibition activities of crude extracts against cancer cell lines Jurkat and HEK293

<i>Xylaria</i> Isolates.	IC ₅₀ (µg/ml)	
	Jurkat	HEK293
<i>Xylaria</i> sp.1	2.63	23.9
<i>Xylaria</i> sp.2	81.3	2.94
<i>Xylaria</i> sp.3	5.26	8.47
<i>Xylaria</i> sp.4	5.89	43.34
<i>Xylaria</i> sp.5	6.13	9.57
<i>Xylaria</i> sp.6	5.39	17.61
<i>Xylaria</i> sp.7	36.69	58.59
<i>Xylaria</i> sp.8	8.86	14.2
<i>Xylaria</i> sp.9	14.13	7.63
<i>Xylaria</i> sp.10	16.9	23.2
<i>Xylaria</i> sp.11	59.21	57.2
<i>Xylaria</i> sp.12	30.76	3.25

Cinnamomum iners belongs to the family of cinnamom (Lauraceae) and is commonly found in Myanmar, Thailand, Malaysia, Indonesia and Philippines. There are many reports about its aroma characteristic and exhibit medicinal properties from their many parts. *C. porrectum* oil also has an antibacterial and antifungal activity. Phongpaichit *et al.* (2006) reported that they extracted oil from the root of *Cinnamomum porrectum* which were tested for its antimicrobial activity. [24] Gu and Ding (2008) reported that two new tetralone derivatives named Xylariol A and B [25] were isolated from ethyl acetate extract of the culture broth of *Xylaria hypoxylon* AT-028. It showed moderate cytotoxic activities against hepatocellular cell line (Hep G2 cells) in the *in vitro* cytotoxic assay with IC₅₀ values.

4. Conclusion

Sixty-four isolates belonging to 12 genera of *Xylaria* were classified to each genus by using morphological characteristic. From the results in this study, *Xylaria* sp. isolates 1 and 2 isolated from *Cinnamomum iners* showed strong antimicrobial and cytotoxic activity. It can be concluded that *Xylaria* sp. could be proved as a good source of efficiency bioactive compounds and would be useful as source of drug development.

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