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Antibacterial and Anticancer Activity of Stromatic *Xylaria* spp. from Tropical Forest Thailand

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Abstract

This research is part of a bioactive metabolite study of fungi collected from Trat Agroforestry Training Station, Trat province, Thailand. The ultimate goal is to screen for bioactivity, including those exhibiting antibacterial and anticancer, activity from isolates of stromatic *Xylaria* spp. Fresh specimens of *Xylaria* spp. were cultured on Potato Dextrose Agar (PDA) for 14 days, and then transferred to Malt Extract Broth (MEB). All isolations were cultured under static conditions for 6 weeks and extracted with ethyl acetate and concentrated by evaporation. All crude extracts were tested for antimicrobial activities against 4 pathogenic microorganisms, including Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), and Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*), in terms of MIC values. Anticancer activity against 5 human cell lines was measured by MTT colorimetric assay. The results showed that all crude extracts inhibited at least 1 tested microorganism. Ten of the extracts showed a broad spectrum of action against the test bacteria. Selected *Xylaria* isolates were chosen for examination of their anticancer activity. Isolate TR 25 showed the highest cytotoxicity; this was against KATO-III cell line with a IC_{50} value of 7.8 $\mu g.mL^{-1}$.

Keywords: *Xylaria*, stromata, bioactive metabolites, antimicrobial, anticancer

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1. Introduction

Currently drugs derived from natural sources including plants, microorganisms, and marine organisms are widely accepted and significantly used for prevention and treatment of human diseases [1- 2]. Metabolites from microorganisms have been considered as potentially new sources and there has been a great deal of research carried out on a wide range of fungi. The fungi are considered to be an excellent source with high potential as a resource of novel bioactive metabolites. It has been estimated that there are over 5 million species of fungi but only 14% have to date been discovered [3-5].

The Xylariaceae are one of the largest and relatively well-known fungal families belonging to the Ascomycota and they exhibit a worldwide distribution being especially well represented in the tropics and subtropics [6-9]. Moreover, members of the Xylariaceae have received considerable attention as sources of secondary metabolites [10-11]. *Xylaria* Hill ex Schrankis one of the largest of the genera belonging the Xylariaceae and is commonly found as saprophytes and endophytes with a number, which are phytopathogenic [11]. Many of them are able to produce metabolites active against agents causing human diseases and include anti-cancer [12], antifungal [13], antioxidant [14], antimicrobial [15], anti-inflammatory [16], and anti-viral activities [17].

Thailand is considered one of the areas containing a high percentage of unknown taxa of Xylariaceae [18-20] andit is reasonable to expect a large number of interesting metabolites to be isolated from *Xylaria* species occurring there. Animportant objective of this study ~~is~~was therefore to screen for bioactive metabolites from stromatic *Xylaria* spp., collected from Trat Agroforestry and Training Station at Trat province.

2. Materials and Methods

2.1 Fungal material

Stromata of *Xylaria* spp. were collected from a tropical rain forest in Trat province, Thailand, identified and authenticated on the basis of stomal morphological characteristics. The ascospores of fresh *Xylaria* specimens were isolated and cultivated on Potato Dextrose Agar (PDA) for 2 weeks.The cultures from first isolated were subcultured several times until the pure culture were obtained. After that,the pure cultures of *Xylaria* spp. were grown on Potato Dextrose Agar (PDA) at room temperature (25-30°C) for 7-14 days depending on their growth rate.

2.2 Fermantation and Extraction

Seven-day-old cultures of the *Xylaria* spp. on PDA were transferred to Malt Extract Both (MEB) in 500 mL Erlenmeyer flasks, followed by incubation at room temperature (25-30°C) under static conditions for 6 weeks. The fermentation broths were filtered through a filter paper and extracted three times with ethyl acetate. Each organic phase was then pooled and concentrated by rotary evaporation. Dried crude extracts were prepared for examination of their biological activities by dissolving in dimethyl sulphoxide (DMSO).

2.3 Antibacterial activity

For primary screening reference pathogenic microorganisms were used for this assay including two Gram-positive bacterial strains (*Staphylococcus aureus* and *Bacillus subtilis*), two Gram-negative bacterial strains (*Pseudomonas aeruginosa* and *Escherichia coli*). The Minimum Inhibitory Concentration (MIC) values of the extract against the test microorganisms were determined by broth micro-dilution method as recommended by CLSi [21]. The test crude extracts in DMSO (25.6 $mg.mL^{-1}$) were diluted with Mueller-Hinton broth (MHB). The concentration of

all crude extracts was examined using two-fold dilution method starting from 4 $mg.mL^{-1}$ to a final concentration of 0.064 $mg.mL^{-1}$. Fifty microliters of MHB containing the test crude extracts were dispensed in each well of microtiter plates (96-well plates) for the evaluation of antibacterial activities. Sterile crude extract-free medium containing the corresponding amount of DMSO was dispensed in the control wells. The finally adjusted bacterial suspensions were inoculated into each well with the volume of 50 µL. Crude extract-free MHB (100 µL) was used as the sterility control and the experiments were carried out in triplicate.

2.4 Anticancer activity

The cytotoxic effect of fermentation broth on anticancer activity was tested by the MTT assay. The five human cancer cell lines including breast cancer (BT474), Human colorectal adenocarcinoma (SW620), Human gastric carcinoma (KATO-III), Human liver hepatoblastoma (HepG2) and Human acute T cell leukemia (Jurkat) cell line were investigated in this research. The experimental protocol was followed as described by Palaga *et al* [22]. Cancer cell lines were maintained in RPMI-1640 medium containing 10% Fetal Bovine Serum (FBS), 100U/ml penicillin, 0.4 mg/ml streptomycin, 1% sodium pyruvate and 1% HEPES. They were seeded in 96-well plates ($1x10^5$ cell/ml in 100µl culture) and incubated at 37° C in a humidified atmosphere with 5% CO_2.

Cell lines were treated with crude extract for 24 hrs. Four hours before the end of the treatment, MTT solution (5mg/ml; 10 µl) was added and incubated until the end of treatment. At the end of treatment, 0.4 N HCl in isopropanol was added to dissolve formazan and the absorbance was measured using a micro-culture plate reader at 540 nm. Percent of cell viability (%) was calculated using the following formula

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

2.5 Statistical analysis

All the analyses were carried out in triplicate. Moreover, The data was presented as means ± standard deviations.

3. Results and Discussion

The objective of this study was to screen for bioactive compounds extracted from isolated stromatic *Xylaria* species and included antimicrobial and anticancer activities. The ascospores of a number of different *Xylaria* species collected from Trat Agroforestry Training Station were isolated, germinated and cultivated on Potato Dextrose Agar (Figure 1). The different isolates were then inoculated by transfer of mycelium from the edge of actively growing colonies into flasks containing 500mL of Malt Extract Broth and incubated for 30 days. Broth solutions were filtered using a membrane filtration method to separate the mycelium from the filtrate and were then extracted with ethyl acetate. The solvent phase was evaporated by rotary evaporation. Crude extracts of 10 isolates were dissolved in 10% DMSO for testing their biological activity.

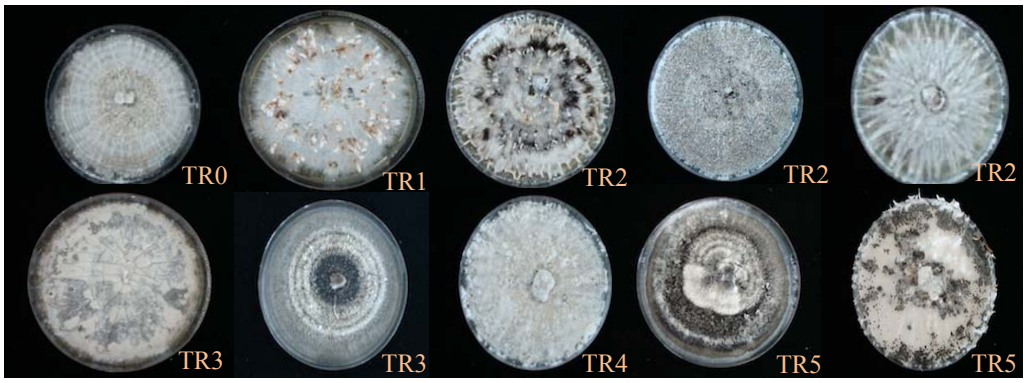

Figure 1 Colony morphology of Xylaria spp. isolated on PDA plates for 2 months

For the primary screening antibacterial activity was used as the criteria for more detailed investigations. The preliminary antimicrobial assay of all crudes extractsagainst *Staphylococcus aureus, Bacillus subtilils*, *Escherichia coli*, and *Pseudomonas aeruginosa* were carried out by MIC values. The determination of minimum inhibitory concentrations (MIC) is often used as the best methodology for evaluating the resistance of bacteria to antibiotics [23]. The results present the lowest concentration of crude extract showing complete inhibition of growth.

According to the results in Table 1 *Xylaria* sp. TR 15 displayed the strongest antibacterial activity among these samples, inhibiting *Staphylococcus aureus,* with MIC less than 0.064 mg. mL^{-1}. Ten isolates (TR01, TR15, TR23, TR25, TR28, TR30, TR31, TR34, TR51, and TR54H) showed broad spectrums against all bacterial strains. The percentage of *Xylaria* sp. isolates exhibiting antimicrobial activity against *Staphylococcus aureus* (92.85%), *Bacillus subtilis* (81.48%), *Escherichia coli* (59.25%) and *Pseudomonas aeruginosa* (51.85%), respectively.

The results revealed that crude extracts of *Xylaria* spp. showed great activity against four indicator microbes. Moreover, they showed antibacterial activity against Gram positive bacteria more than Gram negative bacteria. Yao and Moellering [24] explained that the differences of their activities might 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. The results indicated that *Xylaria* spp. displayed a moderate antibacterial activity. According to Liu *et al* [25], an endophytic *Xy-laria* sp. isolated from *G. biloba* has been shown to have a broad antimicrobial activity. The samples that showed significant antimicrobial activity was further studied for their other biological activities such as anticancer activity.

Table1 The antimicrobial activity measured by the MIC determination

Isolate	MIC values (**$\mu g.mL^{-1}$**)			
	STA	BAS	ESC	PSA
TR01	>256	>2	>2	>4
TR02/1	>2	>2	NA	NA
TR02/3	>2	>2	NA	NA
TR03/1	>2	>2	NA	NA
TR04	>2	>2	NA	NA
TR08	>1	NA	NA	NA
TR09X	>1	>2	>2	NA
TR15	>64	>256	>1	>2
TR17	>2	NA	NA	NA
TR20	>1	NA	>1	>2
TR23	>1	>1	>1	>1
TR25	>128	>256	>1	>2
TR28	>256	>512	>4	>4
TR30	>2	>1	>2	>1
TR31	>256	>256	>256	>1
TR34	>1	>1	>1	>1
TR35	NA	>2	NA	NA
TR36	NA	>2	NA	NA
TR42X	>512	>512	>2	>256
TR46	>512	>512	NA	NA
TR47	>1	>1	>1	>1
TR48	>2	NA	>2	NA
TR49A	>1	>1	>1	>2
TR51	>512	>1	>1	>2
TR54H	>256	>1	>1	>2
TR55	>4	NA	NA	NA

Refer to Table 1 for pathogenic microorganism test code **STA** = *Staphylococcus aureus*, **BAC** = *Bacillus subtilis*, **ESC** = *Escherichia coli*, **PSE** = *Pseudomonas aeruginosa*

The ten selected crude extracts were examined for cytotoxicity against 5 human cancer cell lines [breast cancer (BT474), Human liver hepatoblastoma (HepG2), Human acute T cell leukemia (Jurkat), Human gastric carcinoma (KATO-III), and Human colorectal adenocarcinoma (SW620) cell lines] by MTT assay and used Doxsorubicin (DOX) as the positive control. From the results obtained four fungal isolates including, TR25, TR30, TR34, and TR51 displayed strong specific cytotoxicity toward one or two cancer cells with cell viability below 50%. In contrast, all isolates showed low cytotoxic effect toward HepG2. Therefore, *Xylaria* TR25 possessed the highest anticancer activity against KATO-III and showed a stronger anticancer activity than the positive control (Table 2). From related studies, *Xylaria hypoxylon* produces tetralone derivative,

xylariol A and B, which indicated cytotoxicity against HepG2 [26]. Isopimarane diterpene glycosides produced by *Xylaria polymorpha* showed cytotoxicity against human cancer cell lines [27]. It is accepted that different cell lines might exhibit different sensitivities when treated with different extracts. Cell type cytotoxic specificity of the extracts is likely to be due to the presence of different classes of compounds in the extract [28]. Thus, the anticancer activity screening often uses more than one cell line.

Table 2 Percentage of cell viability of crude extracts against all cancer cell line

Isolate	% Cell viability				
	BT474	HepG2	Jurkat	KATO-III	SW620
TR01	79.16±2.35	69.18±2.41	72.46±3.2	76.36±2.51	61.14±2.39
TR15	98.25±5.03	127.39±2.34	99.63±2.88	135.99±4.54	116.30±3.08
TR23	54.80±4.39	73.46±5.55	74.52±3.38	113.74±2.99	84.89±1.1
TR25	57.72±4.82	72.51±5.17	61.74±1.73	37.05±3.67	37.66±3.28
TR28	101.47±3.42	79.20±2.79	70.33±1.28	75.05±4.56	57.98±0.98
TR30	79.32±1.73	53.28±0.32	49.99±1.86	44.83±1.35	38.55±4.16
TR31	98.51±4.97	117.98±0.69	88.68±1.93	104.84±3.51	118.73±2.53
TR34	70.35±3.51	69.63±4.99	63.57±0.76	48.48±3.16	54.22±1.06
TR51	43.49±3.89	65.23±4.64	47.36±2.58	57.15±1.33	53.01±1.34
TR54H	115.97±3.72	116.66±3.62	84.69±0.42	126.20±5.32	116.67±1.43
Doxolubicin	45.62±1.32	35.19±3.31	11.18±0.91	43.72±1.16	19.44±2.67

Figure 2 shows the anticancer activity of the crude extracts TR25 compared with DOX. The results demonstrated anticancer activity in a dose-dependent manner. The corresponding IC_{50} values for anticancer activity are presented at 7.787 μg mL^{-1}.

Xylaria is a large genus well known as potent sources of bioactive compounds. Previous studies regarding metabolites of *Xylaria* species have demonstrated the presence of several groups of natural products such as phenolics and alkaloids, which were possessed cytotoxicity with cancer cell lines. Thus, the biological activities reported in this study are in broad agreement to those published.

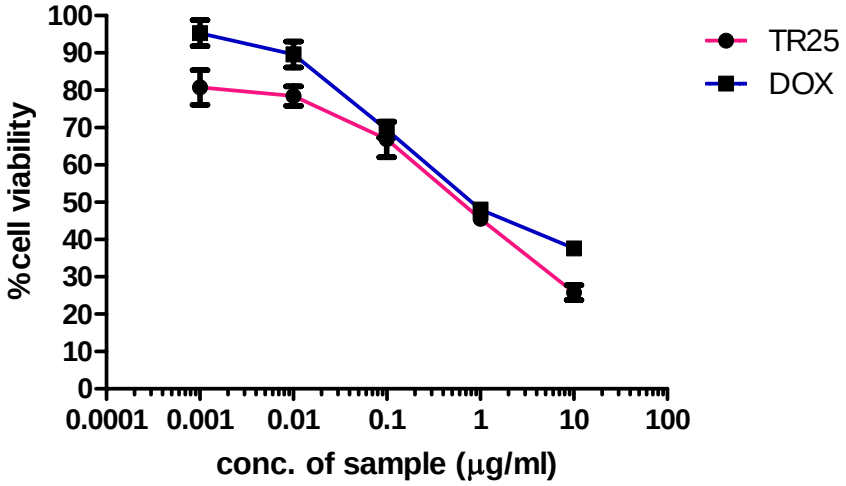

Figure 2 Percentage of cell viability of crude extracts TR25 compared with positive control (DOX) against KATO III cell line

4. Conclusions

The objectives of this study were to isolate stromatic *Xylaria* species and to screen for bioactive compounds derived from their metabolites produced in liquid culture and included antibacterial and anticancer activities. Some of the isolated compounds showed a number of biological activities with significant MIC or IC_{50} values compared to the control drugs. Based on this preliminary work, it can be concluded that the *Xylaria* sp. TR25 is a potential sources of anti-bacterial and anti-cancer compounds. Further studies are necessary to investigate the chemical constituents of the crude extract.

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