

Full Length Research Paper

Antimicrobial and anticancer activities of endophytic fungi from *Mitrajyna javanica* Koord and Val

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Accepted 20 September, 2013

A study was conducted for screening the antimicrobial and anticancer activities of endophytic fungi isolated from leaves of *Mitrajyna javanica* Koord and Val. A total of 73 fungal endophytes were obtained. Fifty-five isolates (75.47%) showed antimicrobial activities against at least one tested microorganism by the paper disc diffusion method. Out of these isolates, 5.47% displayed a broad antimicrobial spectrum. The endophytic fungus *Nodulisporium* sp. PT11 exhibited the strongest antimicrobial activity against all test microorganisms. Anticancer activities were examined with six human cancer cell lines including A375, Katolll, SW620, HepG2, BT474, and Jurkat by the MTT method. The crude extracts of the endophytic isolates exhibited 53.42, 60.27, 54.79, 54.79, 36.98 and 19.17% cytotoxicity (cell viability < 40%) against A375, Kato III, SW620, BT474, HepG2 and Jurkat, respectively. The crude extracts of *Phoma* sp. PT01 had the most specific activity against Jurkat by inducing DNA fragmentation and the characteristic morphological features of apoptosis toward the target cancer cells using Hoechst 33258 fluorescence stain. These results of antimicrobial and anticancer screening support the view that endophytic fungi from *M. javanica* are a potential source of bioactive compounds.

Key words: *Nodulisporium*, *Mitrajyna javanica*, *Phoma*, apoptosis.

INTRODUCTION

Worldwide human health is threatened by cancers and various infectious diseases (Guo et al., 2008; Wang et al., 2008; Tran et al., 2010). The increasing drug resistance to infectious pathogens as well as undesirable effects of certain antimicrobial agents show that there is a desperate need for new and effective bioactive compounds with novel modes of action.

Endophytes are microorganisms that colonize internal plant tissue and can live there for all or part of their life cycle without causing any apparent damage or disease (Petrini, 1991). Nearly one million endophytic species are ubiquitously present in all plants (Amirita et al., 2012). The relationship between the plant and its endophytes is symbiotic in nature (Tran et al., 2010). Endophytic fungi,

particular from higher plants have proven to be a rich source of bioactive and novel organic compounds with interesting biological activities and a high level of biodiversity (Schulze and Boyle, 2005; Tan and Zou, 2001; Li et al., 2009). Several studies indicated that endophytic fungi isolated from medicinal plants have the ability to produce secondary metabolites for antimicrobial and anticancer activities (Wiyakruta et al., 2004; Li et al., 2005; Lin et al., 2007). *Mitrajyna javanica* Koord and Val, belonging to the family Rubiaceae is commonly known as "Krathumna" and is found in all regions of Thailand. Plants of the genus *Mitrajyna* have been used in local folklore medicine for a wide variety of diseases such as fever, malaria, diarrhea, cough and muscular pain (Gong

et al., 2012). It has been used as a Thai medicinal plant for various pharmacological activities. Their leaves have been used for the treatment of diarrhoea, dysentery and frambesia, and the bark for treatment of all types of skin disease, cancer and dysentery (Pongboonrod, 1979). It has been reported that endophytic fungi could produce metabolites similar to or with more activity than their respective hosts (Strobel, 2002; Amirita et al., 2012). Therefore, the Thai medical plant *M. javanica* may constitute another source of endophytic fungi with biological activity. The aim of this study was to evaluate the antimicrobial and anticancer potential of endophytic fungi isolated from *M. javanica*.

MATERIALS AND METHODS

Endophytic fungi isolation

Healthy leaves of *M. javanica* were collected from Ayuthaya (14°21'06"N, 100°34'E) and Pathumthani provinces of Thailand (14°00'48"N, 100°31'49"E) in the wet season. Twenty (20) plants were selected and from each plant 30 leaves were selected. The leaf samples were kept in plastic bags and stored at 4°C in a refrigerator prior to isolation of endophytic fungi. The leaf samples were washed under running tap water for 10 min and then air-dried. Four discs (6 mm in diameter) were cut from the central vein of each leaf and surface sterilized by immersion in 95% ethanol for 1 min, followed by 3.5% (v/v) sodium hypochlorite solution for 3 min and finally 70% (v/v) ethanol for 30s. The surface sterilized samples were washed twice in sterile distilled water and allowed to surface dry on filter papers under sterile condition. The leaf discs were placed on the surface of malt extract agar (MEA) plates supplemented with 150 µg/ml of streptomycin to prevent bacterial growth and incubated at room temperature (30-37°C). The fungal mycelium growing out from leaf discs were subsequently transferred to fresh MEA plates by hyphal tip transfers (Strobel et al., 1996) and incubated at room temperature for 7-14 days. The purity of isolated endophytic fungi was checked and their antimicrobial and anticancer activity determined. The endophytic fungal isolates were maintained in MEA for future studies.

Endophytic fungal identification

The endophytic fungal isolates were identified according to their macro and microscopic structures. The taxa were assigned to genera following Barnett and Hunter (1998), Ainsworth et al. (1973), Ellis (1976) and VonArx (1978). The fungal isolates that failed to sporulate were categorized as mycelia sterilia. The endophytic fungal which displayed the strongest antimicrobial and anticancer activities were identified based on analysis of nucleotide sequences of the internal transcribed spacer (ITS) regions of rDNA. The methods and reagents for DNA extraction and PCR amplification of ITS regions have been described by Rogers and Bendich (1994). The PCR amplification was conducted using primers ITS1 and ITS4. The nucleotide rDNA sequence of ITS was used as the query sequence to search for similar sequences from GenBank using BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Endophytic fungal cultivation and extraction

The endophytic fungi were cultivated on MEA at 30°C for 10 days. Five discs (6 mm diameter) were cut from the edges of growing

cultures and inoculated into 500 ml Erlenmeyer flasks containing 300 ml of malt extract broth (MEB) containing 2% glucose, 0.1% peptone, and 2% malt extract (Merck) and incubated at room temperature for 30 days under static conditions. The filtered broth was extracted exhaustively with ethyl acetate. The extract was dried over anhydrous sodium sulfate and then evaporated under vacuum in a rotary evaporator, to yield ethyl acetate extracts. The crude extracts were then dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at 4°C as stock solution for antimicrobial and anticancer bioassays.

Antimicrobial activity assay

Crude extracts were screened for their antimicrobial activity by the paper-disc diffusion method (Wang et al., 2007). The *in vitro* antimicrobial susceptibility test was performed using the following reference microorganisms; *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 25922, *Saccharomyces cerevisiae* TISTR 5169 and *Candida albicans* ATCC 1023. The plain filter paper discs (6 mm diameter) were impregnated with crude extract solutions and placed on inoculated plates. Streptomycin (50µg/disc, BBL) and Nystatin (100 units/disc, BBL) were used as positive controls. DMSO was used as a negative control. The paper disc was air dried and placed on the bacterial and yeast inoculated plates incubated at 37°C for 24 h. The antimicrobial activities were evaluated by measuring the diameter of the inhibition zones in millimeters. The experiment was replicated 3 times.

Anticancer activity assay

The endophytic fungal crude extracts were screened for their anticancer activities against six human cancer cell lines A375 (malignant melanoma), Kat0III (gastric cancer), SW620 (colorectal cancer), HepG2 (liver cancer), BT474 (breast cancer), and Jurkat (T-cell leukemia) (the cell lines were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Thailand), by MTT method (Palaga et al., 1996). The cancer cells were harvested from exponential phase maintenance culture (T-75 cm²) in RPMI 1640 Medium and seeded in 96 well microculture plates, 100 µl/well (5x10⁴ cells) incubated for 24 h at 37°C, with 5% CO₂ and at 95% relative humidity. A 5 µl of crude extract was added into each well of 96 well microculture plates and incubated at 37°C, with 5% CO₂ and at 95% relative humidity for 24 h. Each sample was tested in triplicate. At the end of exposure, 10 µl of 5 mg/ml (w/v) of MTT solution was added to each well and incubated for 24 h at 37°C, with 5% CO₂ and at 95% relative humidity and then the supernatant was removed and 100 µl of isopropanol containing 0.04 N HCl was added to solubilized the MTT-formazan crystal. The optical densities of the wells were measured with a microplate reader at 540 nm. Cell viability was calculated by the following formula: Viability (%) = (OD_t - OD_b)/(OD_c - OD_b)x100; where, OD_t, optical density of the treated; OD_b, optical density of blank and OD_c, optical density of control.

Cytotoxicity of endophytic fungi by apoptosis

The active anti-cancer crude extracts were tested for inducible apoptotic cell death (DNA fragmentation) detected by staining of DNA with Hoechst 33342 fluorescence dye. The cell lines suspension at 1x10⁶ cells/ml were cultured on the coverslip in 35 mm Petri dishes and incubated at 37°C, with 5% CO₂ and at 95% relative humidity for 24 h. Subsequently, the cells were treated with 50 µl of crude extract. Negative control cells were treated with 0.1%

Table 1. Colonization frequency of endophytic fungi isolated from *M. javanica* leaves.

Endophytic fungi	% Colonization frequency (CF)	
	Ayuthaya	Pathumthani
<i>Alternaria</i> sp.	2	1
<i>Aspergillus</i> sp.	3	-
<i>Cladosporium</i> sp.	1	1
<i>Collectotrichum</i> sp.	3	2
<i>Daldinia</i> sp.	1	-
<i>Fusarium</i> sp.	2	-
<i>Nodulisporium</i> sp.	-	1
<i>Penicillium</i> sp.	2	-
<i>Phomopsis</i> sp.	4	4
<i>Phoma</i> sp.	-	1
<i>Phyllosticta</i> sp.	4	3
<i>Xylaria</i> sp.	3	1
<i>Mycelia sterilia</i>	22	12
Total number of isolates	47	26

DMSO. Positive control cells were treated with 100 µg of etoposide. After that, cells were washed with phosphate buffered saline (PBS) at pH 7.4 and fixed with 1% glutaraldehyde in PBS for 2 h at room temperature under dark conditions. Finally, the cells were washed with PBS and the nuclei were stained with Hoechst 33258 (1 µg/ml in PBS) fluorescence dye and covered with a glass slide. Cell apoptosis were observed under fluorescence microscopy and the fluorescence images were recorded using a digital camera (Fan et al., 2003).

RESULTS AND DISCUSSION

Endophytic fungi

A total of 73 isolates of endophytic fungi were obtained from healthy leaves of *M. javanica*. The endophytic fungal isolates were characterized and identified according to culture characteristics, colony growth and conidia morphology and the best endophytes tested for their biological activities were identified by molecular techniques. Thirty-nine (53.42%) isolates belonging to 12 fungal taxa were identified as *Collectotrichum*, *Fusarium*, *Phomopsis*, *Phoma*, *Alternaria*, *Phyllosticta*, *Xylaria*, *Penicillium*, *Cladosporium*, *Nodulisporium*, *Aspergillus*, *Daldinia* and 34 (46.58%) isolates which did not sporulate were designed as mycelia sterilia (Table 1). Most of the taxa are common endophytic fungi with previous reports (Chareprasert et al., 2006; Gond et al., 2007; Huang et al., 2007).

According to Carvalho et al. (2012), *Collectotrichum*, *Diaporthe*, *Neofusicoccum*, *Penicillium*, *Pestalotiopsis*, *Phomopsis*, *Preussia*, *Pseudofusicoccum* and *Xylaria* were the common endophytes in medicinal plant *Stryphnodendron adstringens*. Species of *Collectotrichum* were the most frequent endophytes. Half of the endophytic fungal isolates were non-sporulating fungi that

grouped into mycelia sterilia. Amirita et al. (2012) reported that the endophytic fungi isolated from four medicinal plants were sterile forms (48%) and were the most when compared to Hypomycetes (25%), Coelomycetes (14%) and Xylariales (13%). This is consistent with previous reports that most of the endophytic fungi did not produce conidia or spores (Saikkonen et al., 1998; Huang et al., 2007; Lin et al., 2007; Gong and Gou, 2009) when cultured on common mycological media (Wiyakrutta et al., 2004). In these studies, the common endophytes *Phomopsis*, *Phyllosticta*, *Collectotrichum*, and *Xylaria* were found in both Ayuthaya and Pathumthani provinces. *Aspergillus*, *Fusarium* and *Daldinia* were found only from Ayuthaya while *Phoma* and *Nodulisporium* were found from Pathumthani. The colonization of endophytic fungi in Ayuthaya was higher than Pathumthani.

Endophytic PT01 and PT11 showed the best biological activities and were subsequently identified by molecular techniques. PT01 exhibited the highest specific cytotoxicity and induced jurkat-human leukemia cells and the PT11 showed the best antimicrobial activity. The ITS sequence of potential cytotoxicity isolate PT01 was closely related to *Phoma herbarum* (99% identity). The ITS sequence of the most potential antimicrobial fungal isolate PT11 was closely related to *Nodulisporium* (99% identity).

Antimicrobial activities of endophytic fungi

The crude extracts of 73 endophytes exhibited a wide variety of antimicrobial activities against 6 tested microorganisms. Fifty-five isolates (75.47%) of endophytic fungi produced bioactive compounds that exhibited antimicrobial activity against at least one test microorganism

Table 2. Antimicrobial activities of crude extract of culture broth of endophytic fungi having broad spectrum and effective antibacterial activity.

Endophytic fungal isolate		Inhibition zone (mm) (mean±SD)				
Code	Strain	BS	SA	EC	PA	CA
AY03	<i>Cladosporium</i> sp.	13.00±0.00	14.00±1.00	14.00±1.00	14.00±1.00	14.00±0.00
AY 07	<i>Mycelia sterilia</i>	12.00±1.00	15.33±0.57	11.66±0.57	11.66±0.57	-
AY08	<i>Mycelia sterilia</i>	19.00±1.15	18.00±0.00	17.00±0.00	20.00±0.00	-
AY 09	<i>Daldinia</i> sp.	9.66±0.57	16.00±0.00	8.66±0.57	8.66±0.57	-
AY 10	<i>Mycelia sterilia</i>	13.33±0.57	17.66±0.57	10.66±0.57	12.33±0.57	9.00±1.00
PT08	<i>Phomopsis</i> sp.	15.00±0.57	24.66±0.57	12.00±0.00	8.66±1.15	20.00±0.00
PT11	<i>Nodulisporium</i> sp.	40.00±1.00	30.00±0.00	20.00±0.00	15.00±0.00	30.00±0.00
	Streptomycin (50µg/disc)	20.00±0.00	20.00±0.00	16.00±0.00	22.00±0.00	-
	Nystatin (100 units/disc)	-	-	-	-	15.00±0.00

BS= *B. subtilis*, SA=*S. aureus*, EC = *E. coli*, PA = *P.aeruginosa*, and CA = *C. albicans*.

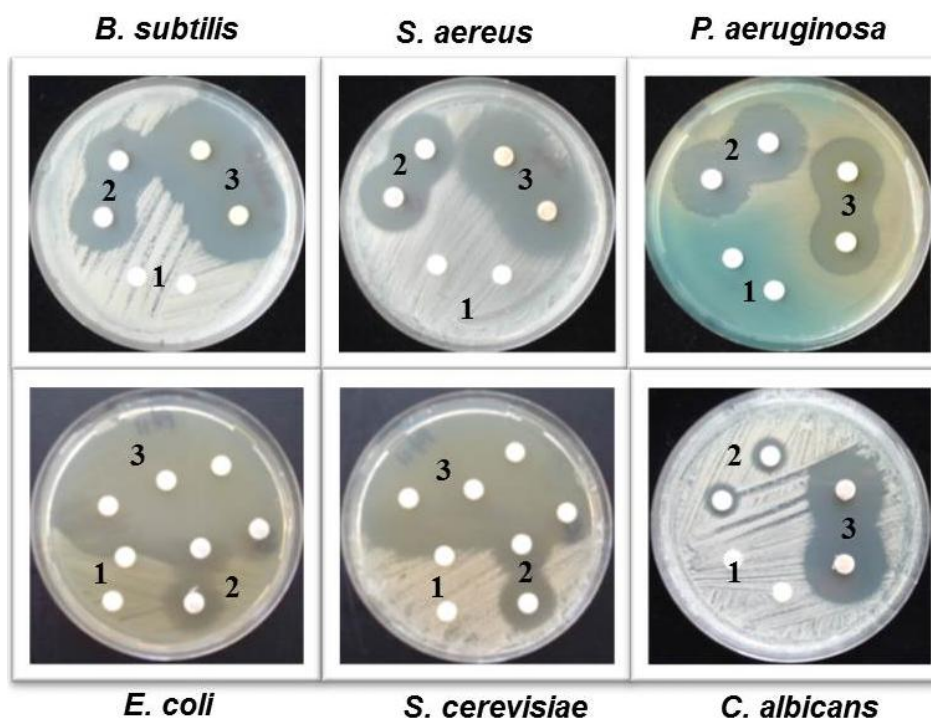


Figure 1. Endophytic *Nodulisporium* sp.PT11 crude extract exhibiting broad spectrum activity, 1 negative control, 2 positive control and 3 treated with crude extract of *Nodulisporium* sp.PT11.

with inhibition zones ranging from 8 to 40 mm. These were 45 (61.64%), 40 (54.80%), 13 (17.80%), 16 (21.92%), 11 (15.10%) and 7 (9.58%) isolates whole crude extracts inhibited *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. cerevisiae*, and *C. albicans* respectively. Four isolates (5.47% such as *Cladosporium* sp. AY03, *Mycelia sterilia* AY10, *Phomopsis* sp. PT08 and *Nodulisporium* sp. PT11 displayed a broader antimicrobial spectrum (Table 2). The endophytic fungal isolate *Nodulisporium* sp. PT11, had strong broad spectrum

antimicrobial activity against all bacteria and the yeast (Figure 1). The Gram-positive bacteria appeared to be more susceptible to the inhibitory effect of the crude extracts than Gram-negative bacteria and the yeast. Rehman et al. (2011) reported that a *Nodulisporium* sp. isolated from *Nothapodytes foetida* showed significant growth inhibition mainly against disease causing Gram positive bacteria. An endophytic *Nodulisporium* sp. isolated from *Juniperus cedrus* produced nodulisporin A-C which exhibited antimicrobial activity against

Table 3. The endophytic fungal isolates having strong specific anticancer activities; shows as a percentage of viability.

Code	Strain	Cancer cell line					
		A375	Kat0III	SW620	HepG2	BT474	Jurkat
AY03	<i>Cladosporium</i> sp.	-	-	10.19±1.61	-	-	-
AY07	<i>Mycelia sterilia</i>	21.03±4.60	-	-	7.11±2.25	-	-
AY16	<i>Penicillium</i> sp.	-	-	7.87±0.53	15.01±0.75	-	-
AY19	<i>Xylaria</i> sp.	-	9.05±1.40	25.08±2.93	-	-	-
AY25	<i>Mycelia sterilia</i>	-	15.53±4.33	-	-	-	-
AY39	<i>Colletotrichum</i> sp.	11.16±2.90	-	-	-	-	-
AY41	<i>Phomopsis</i> sp.	-	11.13±4.22	13.55±3.11	-	-	-
AY45	<i>Xylaria</i> sp.2	-	-	-	11.54±4.14	-	-
AY49	<i>Mycelia sterilia</i>	-	16.95±0.53	-	-	-	-
PT 01	<i>Phoma</i> sp.	-	28±1.65	-	-	-	0.00
PT 26	<i>Phylosticta</i> sp.	16.21±2.69	-	-	-	-	-

-, Cell viability > 40%.

Microbotryum violaceum. (Dai et al., 2006), while this endophyte isolated from *Erica arborea* produced many bioactive compounds such as nodulisporin, 2, 4, 6-trimethyloct-6-ene-3,5-diol, 5-hydroxy-2-hydroxymethyl-4H-chromen-4-one and 3-(2,3-dihydroxyphenoxy)-butanoic acid and exhibited antifungal, antibacterial and anti-algal activity (Dai et al., 2009). Nodulisporacid A was isolated from a marine-derived fungus, *Nodulisporium* sp. CRIF1, exhibited modulate antiplasmodial activity (Kasetrathat et al., 2008). Suwannarach et al. (2013) reported that *Nodulisporium* sp. CMU-UPE34 produced 31 volatile compounds with antifungal activity.

Several preliminary studies have been reported on screening of endophytic fungi from medicinal plants for production of antimicrobial activities found that more than 50% of isolates displayed antimicrobial activity against at least one tested microorganisms (Wang et al., 2007). Gong and Guo (2009) reported that 56% of endophytic fungi from *Dracaena cambodiana* (Agavaceae) inhibited growth of at least one of the test organisms and 8% showed broad spectrum inhibition. Crude extracts of nine endophytic fungi (75%) isolated from *Adenocalymma alliaceum* Miers. showed antibacterial potential against one or more clinical human pathogen (Kharwar et al., 2011).

Most of the bioactive metabolite compounds from endophytic fungi reported were more effective against Gram-positive bacteria than Gram-negative bacteria and pathogenic fungi (Chareprasert et al., 2006). Siqueira et al. (2011) reported that 16 out of 203 endophytic isolates showed antimicrobial activity with a wider action spectrum inhibiting Gram-positive and Gram-negative and fungi.

Anticancer activities of endophytic fungi

The crude extract of 73 fungi displayed anticancer activities

towards six cell lines below 40% of cell viability. The results show that 39 (53.42%), 44 (60.27%), 40 (54.79%), 40 (54.79%), 27 (36.98%) and 14 (19.17%) endophytic fungi exhibited anticancer activities against A375, BT 474, Kat0III, SW620, HepG2 and Jurkat, respectively. Five endophytic fungal isolates exhibited cytotoxicity toward all cell lines. Eleven fungal isolates displayed strong specific cytoactivity toward one or two cancer cell with cell viability below 25% (Table 3). According to Chandra (2012), many endophytic fungi are a novel source of potential anticancer drugs. Several secondary metabolites isolated from endophytic fungi have potential as anticancer activity (Tan and Zou, 2001; Guo et al., 2006 and Chandra, 2012). These are evidence that bioactive compound produced by endophytes could be alternative approaches for discovery of novel anticancer drugs (Selim et al., 2012; Guo et al., 2008).

The most specific active endophytic fungi were evaluated for their potential to induce apoptosis cell death through DNA fragmentation in cancer cells when stained with Hoechst 33258 fluorescence stain. DNA fragmentation (apoptotic body) in particular has been used as an indication of apoptosis when observed under fluorescence microscopy. After treatment with each crude extract at a 5% (v/v) final concentration, the cancer cells morphology were determined. Six isolates produced apoptotic bodies when observed by fluorescence microscopy, indicating that these crude extracts had induced apoptosis.

After treatment of jurkat-human leukemia cells with *Phoma* sp. PT01 crude extract for 24 h, the result showed that *Phoma* sp. PT01 induced DNA fragmentation morphological features of apoptosis of about 90.04%. Whereas, *Penicillium* sp. AY 16 induced 88% apoptotic cell death in human colon cancer (SW620) cells after treatment for 36 h. *Phomopsis* sp. AY41 had apoptosis-inducing activities against the human breast cancer and gastric cancer (Kato III) at 84.80% after

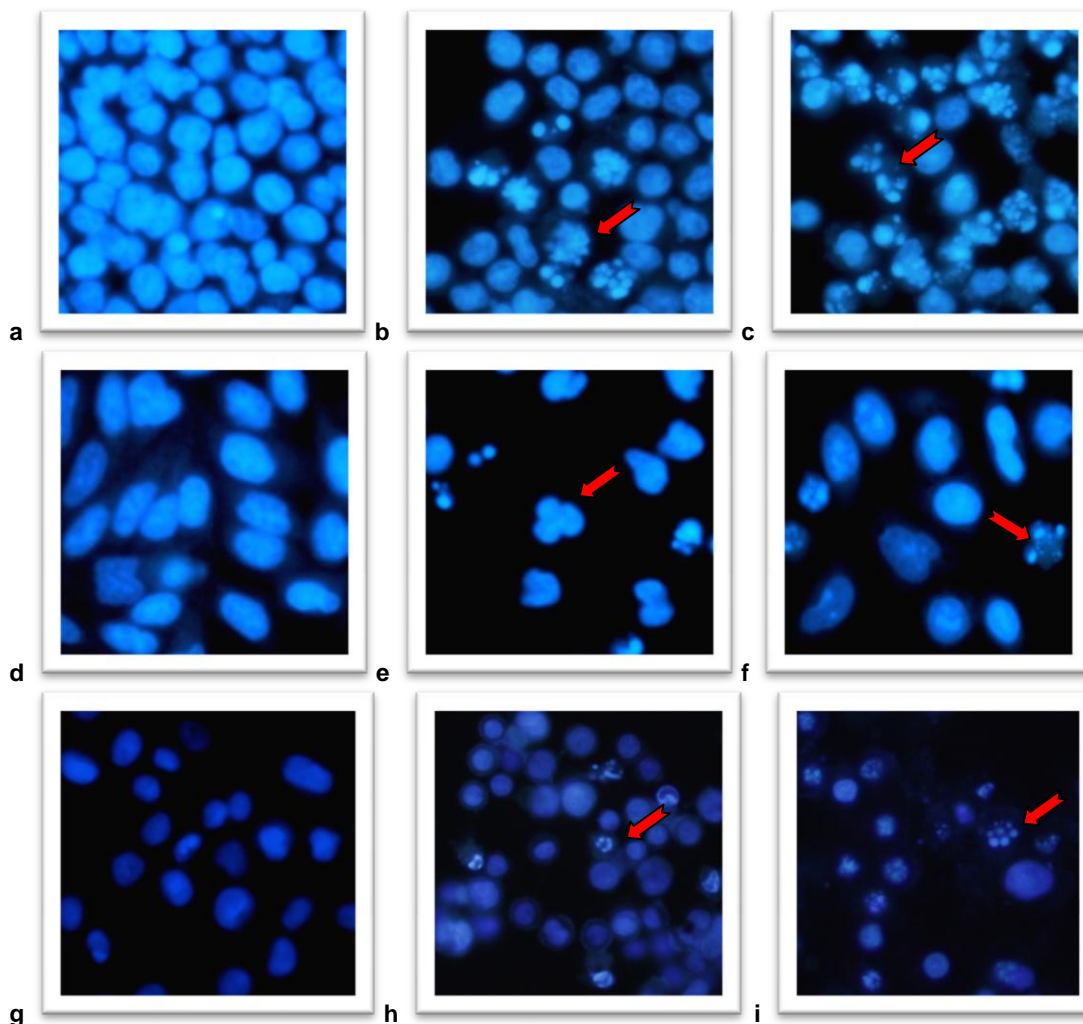


Figure 2. Endophytic fungal crude extracts induced apoptosis in human cancer cell lines; Jurkat (a, b, c), SW620 (d, e, f) and Kato III (g, h, i). Image a, d, and g show untreated cells; b, e, and h treated with crude extract of isolate *Phoma* sp. PT01, *Penicillium* sp. AY16 and *Phomopsis* sp. AY41, respectively; c, f, and i treated with etoposide. Treated cells display nuclear fragmentation and apoptotic bodies (arrows). Magnification x400.

incubation for 24 h. The other isolates did not show apoptosis inducing activity, thus they may have the ability to cause anti-proliferation of cancer cells by necrosis.

The results from fluorescence microscopy of Jurkat, Kato III and SW620 cells after treatment with crude extracts of *Phoma* sp. PT01, *Penicillium* sp. AY 16 and *Phomopsis* sp. AY41 induced cell morphological changes such as cell shrinkage, DNA fragmentation and formation of apoptotic bodies when compared to the cancer cells treated with etoposide as the positive control. Contrastingly, DNA of cells treated with 0.1% DMSO (v/v) and untreated cells (negative control) were visible as faint blue circles and did not show these apoptotic characteristics (Figure 2). *Phoma* sp. PT01 showed the most specific cytotoxicity and towards Jurkat cells. Several studies have reported that *Phoma* species are a potential source of bioactive metabolites. Wang et al. (2012)

isolated cercosporamide, a compound produced by an endophytic *Phoma* isolated from the Chinese medicinal plant *Arisaema erubescens*. It exhibited cytotoxic activity against HT-29, SMMMC-7721, MCF-7, P338, HL-60, and MGC80 cells with IC_{50} values of 9.3 ± 92.8 , 27.87 ± 1.78 , 48.79 ± 2.56 , 37.57 ± 1.65 , 27.83 ± 0.48 , and 30.37 ± 0.28 μ M, respectively. Epoxyphomalins A and B produced by a marine-derived fungus *Phoma* sp. also displayed cytotoxicity at nanomolar concentrations toward human 12 tumor cell lines (Mohamed et al., 2009). *Phoma medicaginis* associated with medicinal plants *Medicago sativa* and *Medicago lupulina*, produce the antibiotic brefeldine A, which also initiated apoptosis in cancer cells.

Apoptosis is an energy-dependent mode cell death requiring active participation of the target cell (Gangadevi and Muthumary, 2007). Additionally, Taxol is an important

anticancer drug used widely in the treatment of human malignancies, particularly ovarian and breast cancer and its mode of action was revealed by induced apoptotic cell death. Agents that suppress the proliferation of cancer cells by inducing apoptosis may represent a useful mechanistic approach to both cancer chemoprevention and chemotherapy preventing unfavorable side effects and resistance (Shafi et al. 2009). Wang (2011) also reported that defects along apoptotic pathways play a crucial role in carcinogenesis and that many new treatment strategies targeting apoptosis are feasible and may be used in the treatment of various types of cancer. Therefore, there is potential for selected endophytic fungi to produce novel anti-cancer metabolites that specifically target and induce cancer cells to apoptosis.

The results indicate that the endophytic fungi from *M. javinica* with diverse bioactivities in this study are potential drug candidates. The bioactive compounds produced by *Phoma* sp. PT01 and *Nodulisporium* sp. PT11 should be further investigated and characterized.

ACKNOWLEDGEMENTS

The work was supported by 90th anniversary Chulalongkorn University Grant in order to support cooperative research work between Faculty and Graduate student through Rachadapisek Fund. We are thanks for the Institute of Biotechnology and Genetic Engineering (Chulalongkorn University) for supply of the cancer cell lines.

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