Isolation, Characterization and Screening for Anticancer and Antimicrobial Properties of the Crude Extract from Genus *Neosartorya*

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Abstract The genus *Neosartorya*, the sexual states of *Aspergillus*, belongs to the *Eurotiales* which have been reported for compounds with bioactivities such as antimicrobial, antimalarial and cytotoxicity. The objective of this research was to isolate, identify and screening for anticancer and antimicrobial properties of the ethyl acetate crude extract from *Neosartorya*. *Neosartorya* sp. was isolated from soil in Chiang Mai province, Thailand by the soil plate technique. The samples were identified base on morphology and DNA sequencing: Internal transcribed spacer (ITS), β -tubulin and calmodulin as *Neosartorya hiratsukae* and *Neosartorya pseudofischeri*. For in vitro cytotoxicity activity by MTT assay using eight cell lines (MCF-7, P388, HeLa, Vero, L929, KB, HT-29 and HepG2). The crude ethyl acetate extract of *N. hiratsukae* and *N. pseudofischeri* showed the highest potency against the L929 cell line with the 50% inhibitory concentration (IC₅₀) value of 144.31 and 267.73 μ g/ml, respectively. The antimicrobial properties of crude extract were evaluated using disc diffusion method against five bacteria strain: *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The extract was well inhibited the growth of gram positive pathogenic bacteria: *B. subtilis*, *S. aureus* and *M. luteus*. The results indicate its possible potential with the production of other bioactive agents for future anticancer and antibiotic drug development.

Keywords Antimicrobial, Cytotoxicity, *Neosartorya hiratsukae*, *Neosartorya pseudofischeri*

1. Introduction

The genus *Neosartorya* belongs to *Trichocomaceae* family under the order *Eurotiales*. These fungi are perfect state (teleomorphic stage) of *Aspergillus*. Some species of

the genus *Neosartorya* have been reported for compounds with bioactivities such as antibacterial and cytotoxicity. For example, three new compounds isolated from the *Neosartorya pseudofischeri* inhibited the growth of six human cancer cell lines that a quinazolinone displayed cytostatic effects in human U373 glioblastoma and A549 non-small cell lung cancer apoptosis-resistant cells with marked inhibition of mitotic rates [1]. New compounds, a pyrroloindole and fischerindoline, inhibited the growth of six human and mouse cancer cell lines [2]. Including, a new meroterpenoid: tatenic acid was isolated from the fungus *N. tatenoi* exhibited cytotoxicity against human small cell lung cancer (NCI-H187) and human epidermoid carcinoma in the mouth (KB) [3]. Additionally, the crude mycelial extracted from *N. spinosa* were reported as antibacterial [4]. The screening for bioactive compounds from the *Neosartorya* is challenging. Therefore, this research focuses on isolation and identification of *Neosartorya* from the forest soil in Thailand and screening for anticancer and antimicrobial properties.

2. Materials and Methods

2.1. Fungus Isolation and Morphological Characterization

The genus *Neosartorya* were isolated from soil collected at Doi Suthep and Doi Inthanon mountains (Chiang Mai, Thailand). The soil samples were cultured on glucose ammonium nitrate agar (GANA) by soil plate technique [5]. Then, single colony of *Neosartorya* was transferred to PDA for purification. The fungi were grown on PDA about 2 weeks at 25°C for morphological characterization. The identification was done by observation of colony and mycelia morphology, cleistothecia, asci and ascospore using light microscopy and scanning electron microscopy [5-6].

2.2. DNA Extraction, Amplification and Sequencing

Fungal genomic DNA was obtained from 10-15 days old culture mycelia of the potato dextrose broth medium (PDB) and genomic DNA extracted using the standard CTAB method. The ITS ribosomal DNA regions were amplified by PCR using the universal primers, ITS1 and ITS4 [7]. The β -tubulin gene was amplified using the Bt2a and Bt2b [8] and the calmodulin gene was amplified using the CF1L and CF4 primers [9]. The PCR products were purified with PCR purified kit (Vivantis, USA). Sequencing was performed at First Base Laboratories, Malaysia. The obtained nucleotide sequences were searched through BLASTN at GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>).

2.3. Fungal Culture and Extraction

The mycelium was taken by vertically cutting 6 mm diameter plugs using a cork borer. The plugs were transferred to 250 ml bottles containing 10 ml of PDB and incubated at room temperature for 1 month. The culture was harvested by filtration through several layers of gauze and mycelia were collected and dried at 45°C for 3 days. The powdered mycelia were extracted with ethyl acetate on a rotary shaker (120 rpm) for 3 days at room temperature and repeated for 3 times. The supernatants were filtered through a Whatman filter paper No. 1 and concentrated using a rotary evaporator at 40°C. The crude extracts were kept in dark bottles under refrigerated conditions until the time of experiment.

2.4. Determination of Anticancer Activity

The HT-29 (Human colon adenocarcinoma), MCF-7 (Human breast carcinoma), P388 (Murine leukemia), L929 (Murine fibroblasts) and Vero (African green monkey kidney) cell lines were a kind gift from Dr. Porntipa Picha, Research Division, National Cancer Institute, Bangkok. The KB (Human mouth epidermal carcinoma), HeLa (Human cervical carcinoma) and HepG2 (Human hepatocellular carcinoma) cell lines were obtained from Scientific Instruments Centre, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok. The cells were maintained in RPMI 1640 medium with 10% (v/v) fetal bovine serum (FBS) and 0.05 µg/ml gentamicin in 5% CO₂ humidified incubator at 37°C. The MTT assay proposed by Mosmann [10] was modified and used to determine antiproliferative activity of extracts. Briefly, exponentially growing cells were seeded onto a 96-well plate and allowed to attach for 24 hours before treatment. The crude extracts were dissolved in dimethyl sulfoxide (DMSO) and sterilized by filtration through 0.22 µm filter. Cells were treated with 100 µl of those crude extracts within a range of final concentrations from 125 to 2000 µg/ml for 20 hours. The fifty microliters of MTT solution (2 mg/ml in phosphate buffer saline) were added to each well and further incubated for 4 hours at 37°C. Twenty five microgram per milliliter of Mitomycin C (MMC) and 1% DMSO final concentration were added as a positive and negative control, respectively.

All the exposure conditions were tested in triplicate and observed for cell morphological analysis. After the reaction, the supernatant were removed and replaced with DMSO: ethanol (1: 1 v/v) to solubilized formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Anthos MultiRead 400, Biochrom, UK). The percentage of inhibition of cell growth was calculated and the 50% inhibitory concentration (IC₅₀) was estimated using GraphPad Prism 5 software.

2.5. Determination of Antimicrobial Activity

The extracts were tested using agar disc diffusion described by Ansari et al. [11]. Five microbial strains: *Bacillus subtilis*, ATCC6633, *Micrococcus luteus* ATCC9341 and *Staphylococcus aureus* TISTR1466 and two gram negative bacteria (*Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853) were cultured in Mueller-Hinton Broth (MHB) and incubated at 37°C for 4-5 hours. The optical density of cells was adjusted to 0.5-0.8 at 600 nm. Each bacterial suspension was swab over the surface of Mueller Hinton Agar (MHA) with a sterile cotton swab. The dried crude extract was dissolved in a methanol. Various concentrations of extracts (0.20, 0.5, 1 and 2 µg/disc) were loaded onto each the 6 mm sterile paper disc, allowed to dry (about 30 min incubation) then was placed onto inoculated plates. Twenty microliters of methanol and 100 µg/ml gentamicin were used as negative and positive control, respectively. Incubated at 37°C for 24 hours, the diameters of the inhibition zone were measured.

3. Results and Discussion

3.1. Isolation, Morphological and DNA Sequencing Characterization

The morphological features were observed as microscopic characters which showed white to creamy colony and cleistothecia, ascospores are globose to broadly ellipsoidal with equatorial furrow and ridges identified as *Neosartorya*. EU06 had a creamy-white colony, cleistothecia are globose (90.7-263.05x91.98-276.76 µm), ascus is globose (9.86-13.63x8.63-13.9 µm) and ascospores are broadly ellipsoidal (3.86-6.81x3.70-6.48 µm) with two equatorial crests (Figure 1). The other (EU13) had a white colony, cleistothecia are globose (274.44-506.11x301.43-526.58 µm), ascus is subglobose (10.70-12.03x11.23-12.08 µm) and ascospores are subglobose (3.54-5.53x4.30-5.59 µm) with narrow equatorial furrow (Figure 2). These indicate that identification of *Neosartorya* sp. not easy based on morphology. Therefore, we further analyzed DNA sequence of the three regions and combined the data with morphological characteristic in order to confirm species. The nucleotide sequences were compared with BLASTn in NCBI Genbank. The three region were amplified that related to the

two species. For EU06, the fragments for ITS, β -tubulin and calmodulin were 606, 545 and 735 bp, respectively identified as *N. hiratsukae*. For EU13, the fragments for ITS, β -tubulin and calmodulin were 588, 553 and 764 bp, respectively identified as *N. pseudofischeri*. The dendrogram of calmodulin gene was demonstrated representative of these fungi (Figure 3). Our result was supported with findings of Sanmanoch et al. [4] who reported the nucleotide sequences from the ITS region is useful and suitable for *Neosartorya* identification. However, identification of *Neosartorya* should be using both molecular and morphological techniques [12].

3.2. Anticancer Activity

Antiproliferative activity of ethyl acetate extract of *N. hiratsukae* and *N. pseudofischeri* were determined using the MTT assay in eight cell lines (HT-29, MCF-7, P388, L929, Vero, KB, HeLa and HepG2) at concentration ranging from 125 to 2000 μ g/ml. Pretreatment with MTT solution, the cells treated with extract were irregular and round shape when observed under inverted phase contrast microscope. MMC used for positive control induced differences cytotoxicity in cell lines (cytotoxicity > 50%: data not shown). The crude extract of *N. hiratsukae* exhibited antiproliferative activity against the top three cell lines; L929, Vero and HeLa, respectively. The ethyl acetate extract

of *N. pseudofischeri* shown against the top three cell lines; L929, HeLa and HT-29, respectively. The results also show that L929 cell line was the most sensitive to the *N. hiratsukae* and *N. pseudofischeri* extracts with IC₅₀ values are 144.30 and 267.73 μ g/ml, respectively (Table 1). Our findings are consistent with the some species of the genus *Neosartorya* such as *N. pseudofischeri*, *N. tatenoi* and *N. spinosa* have been reported for compounds which potent cytotoxicity against the mammals and insect cell lines [1-4, 13].

3.3. Antimicrobial Activity

The crude ethyl acetate extract of *N. hiratsukae* and *N. pseudofischeri* showed antimicrobial activity against three of five tested human pathogenic bacteria. No inhibitory activity against *E. coli* and *P. aeruginosa* was observed. Crude extracts from *Neosartorya* showed well inhibited the growth of gram positive bacteria (*B. subtilis*, *S. aureus* and *M. luteus*) (Table 2). Our result was supported with findings of Sanmanoch et al. [4] who reported to the antibacterial activities of crude ethyl acetate extract from *N. spinosa* KKU1NK1 most strongly inhibited the growth of gram positive pathogenic bacteria (*S. aureus*, *S. saprophyticus*, *S. pneumonia* and *B. subtilis*).

Figure 1. Morphological features of *Neosartorya hiratsukae*, A-B: Colony grown on PDA for 15 day at 25°C, C-D: Cleistothecia, E: Asci exposed from cleistothecium, F: Ascus, G-H: Ascospores observed under light microscope and I: Ascospores as seen by SEM. Bars, 100 μ m (D) and 10 μ m (E-H).

Figure 2. Morphological features of *Neosartorya pseudofischeri*, A-B: Colony grown on PDA for 15 day at 25°C, C-D: Cleistothecia, E: Cleistothecium, F: Asci, G-H: Ascospores observed under light microscope and I: Ascospores as seen by SEM. Bars, 100 µm (D), 20 µm (E) and 10 µm (F-H).

Figure 3. The dendrogram of *Neosartorya hiratsukae* and *Neosartorya pseudofischeri* based on *calmodulin* gene using Neighbor-joining, bootstrap = 1000

Table 1. Cytotoxic activity of ethyl acetate extract of the *N. hiratsukae* and *N. pseudofischeri*

Type	Cell lines	50% inhibitory concentration: IC ₅₀ (µg/ml)	
		<i>N. hiratsukae</i>	<i>N. pseudofischeri</i>
Cancer cell lines	P388	1,464.45	714.21
	MCF-7	1,368.31	1,673.19
	HT-29	1,235.14	702.23
	HepG2	1,783.78	>2,000.00
	HeLa	913.07	461.11
	KB	1,584.39	1,581.27
Normal cell lines	Vero	687.79	1,038.13
	L929	144.30	267.73

Table 2. Antibacterial activity of crude ethyl acetate extracts from *N. hiratsukae* and *N. pseudofischeri* using paper disc diffusion method

Crude extracts	Concentration (µg/disc)	Zone of inhibition (mm)*		
		<i>B. subtilis</i>	<i>M. luteus</i>	<i>S. aureus</i>
<i>N. hiratsukae</i>	2	10.05±0.02	13.94±0.44	8.99±1.26
	1	9.82±0.09	11.98±1.14	8.20±0.98
	0.5	8.17±0.39	10.54±0.80	6.97±0.30
	0.2	7.83±0.12	9.08±0.85	6.63±0.30
<i>N. pseudofischeri</i>	2	11.74±3.70	9.32±0.63	10.32±0.63
	1	9.61±2.80	7.98±0.16	8.91±0.16
	0.5	9.10±2.70	7.18±0.24	7.57±0.24
	0.2	7.50±0.50	6.98±0.29	6.53±0.68
Gentamicin	2	15.42±0.41	18.99±0.76	12.75±0.14

*Values represent mean (±SE) of three experiments each set up in triplicate

3. Conclusions

The genus *Neosartorya* were isolated from forest soil collected at Doi Suthep and Doi Inthanon mountains, Thailand. Identification of *Neosartorya* species are not necessarily based on morphology, it should be used both morphological characterization and molecular methods. Screening for its bioactive compound, the crude ethyl acetate extract of the *N. hiratsukae* and *N. pseudofischeri* were analyzed to eight different mammalian cell lines which showed potent cytotoxic effect in some cell lines. Additionally, the crude extracts inhibited the growth of gram positive bacteria. Our findings provide further evidence that the fungus *Neosartorya* has the tremendous potential of biosynthesis. Therefore, our attempts to isolate *Neosartorya* from different place or sources and screening for their bioactivities in order to development as an anticancer and antibacterial drug.

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