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ORIGINAL ARTICLE

Evaluation of in vitro alpha-glucosidase inhibitory, antimicrobial, and cytotoxic activities of secondary metabolites from the endophytic fungus, *Nigrospora sphaerica*, isolated from *Helianthus annuus*

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Abstract

Purpose This study aimed to evaluate alpha-glucosidase inhibition and antimicrobial activity as well as cytotoxic activity of extracts from the endophytic fungus, *Nigrospora* sp., isolated from leaves of *Helianthus annuus*, which is widely cultivated for food and used as a medicinal plant.

Methods The fungus (TSU-CS003) was identified based on internal transcribed spacer ribosomal DNA sequences and fungal biomass, and fermentation broth was subjected to extraction by solvents (hexane and ethyl acetate). All extracts were tested for their antimicrobial activity, alpha-glucosidase inhibition, and cytotoxicity activity. In addition, the active extract was analyzed by using gas chromatography mass spectrometry (GC-MS)

Results TSU-CS003 was identified as *Nigrospora sphaerica*. The fermentation broth extract (BE) showed strong antimicrobial activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (Gram-positive bacteria) with minimum inhibitory concentration (MIC) values in the range of 16–32 μg/mL and a few yeasts with MIC values ranging from 64 to 128 μg/mL, especially *Talaromyces marneffei* with an MIC value of 4 μg/mL. The effects of BE were observed by SEM. The results showed that this extract affected the cell morphology of *T. marneffei*. The half-maximal inhibitory concentration (IC_{50}) of BE from alpha-glucosidase inhibition was recorded as 17.25 μg/mL and also showed significant cytotoxicity against A549 human cancer cell lines with an IC_{50} value of 22.41 μg/mL. Furthermore, BE was analyzed by using GC-MS and divided into three main compounds, including 5-pentyldihydrofuran-2(3*H*)-one, (Z)-methyl 4-(isobutyryloxy)but-3-enoate, and 2-phenylacetic acid.

Conclusion This was the first report of the endophytic fungus *N. sphaerica* from *H. annuus*. It is a potential source of active metabolites, which gave the strong antifungal activity, antioxidant activity, and cytotoxicity to A549 cancer cell lines.

Keywords *Nigrospora sphaerica* · Secondary metabolites · ITS rDNA sequence

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Introduction

The side effects of common standard agents are a critical point for patients and have become a serious medical concern worldwide. Many side effects of drugs have been reported (Shobha et al. 2014; Al-Dhabi et al. 2015). Thus, there is need to survey new sources of active metabolites. Recently, plants have been screened for their biological activities (Katiyar et al. 2012; Atanasov et al. 2015). Although the discovery of many active metabolites from plants has occurred during the last decade, health problems are still a major concern and effective

approaches are needed. One of the strategies to solve this issue is finding alternative sources of active secondary metabolites.

Endophytic fungi are a natural source of many active secondary metabolites. They belong to two main phyla: the Ascomycota and Basidiomycota. Many species of endophytic fungi from different host plant species were studied, and their biological activities were determined, such as strong antimicrobial activity, antioxidant activity, anticancer activity, and alpha-glucosidase inhibition (Artanti et al. 2011; Jouda et al. 2016). It has been known that some of antibiotic agents were derived from those endophytic fungi (Deshmukh et al. 2015; Martinez-Klimova et al. 2017). *Nigrospora* is a major endophytic fungus in various host plants, which produces active compounds (Zhao et al. 2012; Thanabalasingam et al. 2015; Ebada et al. 2016). Many reports on the phytochemical analysis of *Nigrospora* spp. have been published. Zhao et al. (2012) identified the active chemical components of a crude extract from *Nigrospora* sp. They found griseofulvin, dechlorogriseofulvin, 8-dihydroramulosin, and mellein are the main chemical components which showed antifungal activity against pathogenic fungi (*Botrytis cinerea*, *Colletotrichum orbiculare*, *Fusarium oxysporum*, *Pestalotia diospyri*, *Pythium ultimum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*) with half-maximal inhibitory concentration (IC_{50}) values that ranged from 0.2 to > 100 μg/mL. Furthermore, there are also many reports on the active chemical compounds from *Nigrospora* spp. Thanabalasingam et al. (2015) identified the active metabolites from *Nigrospora oryzae*. They found two new compounds, including phenazine-1-carboxylic acid and phenazine-1-carboxamide, that have been reported as antifungal agents against *Cladosporium cladosporioides*. Metwaly et al. (2014) found nigrosphaerin A, a new isochromene derivative which showed moderate antifungal activity against *Cryptococcus neoformans* with an IC_{50} value of 14.8 μg/mL.

Helianthus annuus belongs to the family Asteraceae. It has been used as a food and medicinal plant for several diseases. *Helianthus annuus* contains many bioactive elements (alkaloids, tannins, saponin, and phenolic compounds) that showed a different composition in each part of plants, such as seed, root, flower, leaf, and stem, as well as different therapeutic properties (Verma et al. 2016). The previous studies isolated bacterial endophytes from *H. annuus* and characterized the production of jasmonates and abscisic in culture medium (Forchetti et al. 2007). The bacterial endophytes showed good antibacterial activity. However, the study of endophytic fungi from this plant has not been done. Thus, this is the first research aimed to evaluate the potential of the endophytic fungus *Nigrospora sphaerica* isolated from leaves of *H. annuus* for alpha-glucosidase inhibitors, and antimicrobial and anticancer activities.

Materials and methods

Sample collection and fungal isolation

Healthy fresh leaves of *H. annuus* were collected from Phatthalung Province, Thailand. Leaf samples were kept in sterile plastic bags and immediately brought to the laboratory for fungal isolation. The fungal isolation procedures followed Supaphon et al. (2018) with slight modifications. Briefly, all leaf samples were cleaned under running tap water, followed by drying with sterile paper towels. Each leaf was cut with a sterile blade into two 3 × 3 cm^2 segments; surface sterilized with 3% sodium hypochlorite (NaOCl) for 1 min, 70% EtOH for 3 min, and 3% sodium hypochlorite for 1 min; and rinsed with sterile distilled water for 1 min. After that, plant samples were further cut into six 0.5 × 0.5 cm^2 segments and placed in potato dextrose agar (PDA) supplemented with penicillin G and streptomycin (50 mg/mL) to suppress bacterial growth. The plates were incubated at room temperature for 7–14 days. During the incubation period, plates were observed every day and the hyphal tip technique was used for fungal isolation (Brown 1924). Individual fungal isolates were transferred to fresh PDA without antibiotics and incubated under the same conditions to obtain pure cultures for fungal identification. All fungal isolates were identified based on their morphological characteristics (Barnett and Hunter 1998). The fungal isolate *Nigrospora* sp. was kept on PDA slants and stored at room temperature for further use.

DNA extraction, PCR amplification, and sequencing

The selected endophytic fungi were cultured in 50 mL of potato dextrose broth (PDB) and incubated at room temperature for 7 days. After that, the fungal mycelia were obtained by the filtration technique. Genomic DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Inc.). Then, the genomic DNA was analyzed with 1% agarose gel electrophoresis in 1% TAE buffer. For molecular analyses, the region of ribosomal gene (internal transcribed spacer ribosomal DNA (ITS rDNA)) was amplified by PCR with the primer pairs ITS5/ITS4 (White et al. 1990). The PCRs using Taq DNA polymerase (Thermo Fisher Scientific, Inc., Massachusetts) followed the manufacturer's procedures. The amplification of 18S rDNA was performed in the T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., California) as follows: initial denaturation at 95 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, annealing at 50 °C for 40 s, and elongation at 72 °C for 1.30 min, with a 20-min final extension period at 72 °C. The PCR products were examined on a 1% agarose gel stained with RedSafe™ (iNtRON Biotechnology, South Korea). The PCR products were sequenced and purified by Macrogen, Inc., South Korea, for direct DNA sequencing.

Phylogenetic analysis using maximum parsimony and maximum likelihood

Nucleotide sequences in this study were compared with the related sequences from GenBank (http://www.ncbi.nlm.nih.gov) following Wang et al. (2017). All sequences were assembled with BioEdit (version 7.2.5) (Hall 2005) and aligned with Muscle program (version 3.8) (Edgar 2004), while the phylogenetic tree was constructed by maximum parsimony (MP) and maximum likelihood (ML) analyses. MP analyses were performed by PAUP* 4.0b10 (Swofford 2002). The result of most parsimonious trees (MPTs) was evaluated, followed by heuristic searches: 100 replicates of random stepwise addition of sequence, branch-swapping algorithm: Tree Bisection Reconnection (TBR) and equal weight characters. Maximum parsimony bootstrap support of the clades was approximated by 1000 replicates (stepwise addition of sequence, 10 replicates of random addition of taxa, TBR branching-swapping algorithm). Additionally, the maximum likelihood and bootstrap analyses were generated on the CIPRES web portal (Miller et al. 2010) through RAxML 8.2.4 (Stamatakis 2014) with the BFGS method to optimize GTR rate parameters. The phylograms were visualized using FigTree v1.4.3 (Rambaut 2016).

Fungal fermentation and extraction

The selected fungal isolate was inoculated into 300 mL PDB in 500-mL Erlenmeyer flasks and incubated at room temperature for 21 days. After that, culture broth was filtered to separate the fermentation broth and mycelia by filtration technique. The samples were extracted as previously described (Supaphon et al. 2018). Briefly, fermentation broth was extracted twice with an equal volume of ethyl acetate and concentrated to dryness under a rotary evaporator at 45 °C. Fungal mycelia were soaked in methanol for 7 days. The methanol layer was concentrated under reduced pressure. Distilled water (100 mL) was added to the extract, and the mixture was then mixed and extracted twice with an equal volume of hexane. The aqueous layer was then extracted three times twice with an equal volume of ethyl acetate, respectively. Three crude extracts were obtained and kept in sterile Eppendorf tubes. They were dissolved with dimethyl sulfoxide (DMSO) to prepare stock solutions (10 mg/mL) and stored at − 4 °C until used.

Antimicrobial activity

Preparation of test microorganisms

Bacteria *Staphylococcus aureus* ATCC25923, a clinical isolate of methicillin-resistant *S. aureus* (MRSA) SK1; *Escherichia coli* ATCC25922; and *Pseudomonas aeruginosa* ATCC27853 were inoculated by streaked on nutrient agar and incubated at 35 °C for 1 day, then five single colonies of each strain were taken into nutrient broth and incubated at 35 °C for 3–5 h in a shaking incubator. Culture broths were then adjusted to 0.5 McFarland standard with 0.85% sterile normal saline solution (NSS) and diluted with Mueller-Hinton Broth (1:200 v/v). Yeasts *Candida albicans* ATCC90028, *C. albicans* NCPF3153, *Cryptococcus neoformans* (flucytosine-sensitive) ATCC90112, and *C. neoformans* (flucytosine-resistant) ATCC90113 were inoculated by streaked on Sabouraud dextrose agar (SDA). The plates were incubated at 35 °C for 1 day for *C. albicans* and at room temperature for 1 day for *C. neoformans*; then, five single colonies of each strain were transferred to Sabouraud dextrose broth (SDB). After that, cell suspensions were adjusted to 2.0 McFarland standard with 0.85% sterile NSS and diluted with SDB to 1:20 (v/v).

Filamentous fungi *Microsporum gypseum* and *Talaromyces marneffei* were subcultured on PDA and incubated at room temperature for 7 days. Spore suspensions were prepared by using a combination of sterile glass beads with 2 mL of NSS, counted using a hemocytometer, and adjusted to 10^4 spores/mL.

Screening, minimum inhibitory concentration and minimum bactericidal concentration, or minimum fungicidal concentration assays

Screening test Antimicrobial activity assays were performed by the colorimetric broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI 2008, 2012). All crude extracts were screened at a final concentration of 200 μg/mL. Briefly, the stock solution of each extract was diluted with sterile culture broth into 1 mg/mL and 400 μg/mL, respectively. Fifty microliters of 400 μg/mL crude extract was added to each well, then a suspension of the test microorganism was added and mixed well before incubation. Plates were incubated at the same conditions for each test strain as the previous step, followed by adding 30 μL of 0.18% resazurin indicator, and were examined after incubation for the completed reaction.

For bacteria and *C. albicans*, the plates were incubated at 35 °C for 3 h, and *C. neoformans* and *M. gypseum* were incubated at room temperature for 1 day. After incubation, a positive result (no growth of test microorganisms) showed a blue color and a negative result (growth of test microorganisms) showed a pink color, while the growth of *T. marneffei* incubated at room temperature for 6 days was directly observed under a stereomicroscope instead of by adding resazurin (Sarker et al. 2007).

Minimum inhibitory concentration and minimum bactericidal concentration or minimum fungicidal concentration assays

The extracts that showed positive results from screening tests were evaluated for minimum inhibitory concentrations (MICs) using serially twofold dilutions of crude extracts with final concentrations of 128–0.25 μg/mL. The lowest concentration of the extract that inhibited the growth of test microorganisms was recorded as the MIC value. For the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) assays, concentrations of the crude extract less dilute than the MIC and the MICs were streaked onto nutrient agar (NA) plates for bacteria and SDA plates for yeast and filamentous fungi and incubated under appropriate conditions. The plates were incubated under appropriate conditions for each test microorganism. The lowest concentration of the extract that showed growth inhibition on culture media was recorded as the MBC value or MFC value. Antibiotics were used as positive inhibitory controls (vancomycin for Gram-positive bacteria, gentamicin for Gram-negative bacteria, amphotericin B for yeasts, and *T. marneffei* and miconazole for *M. gypseum*).

Scanning electron microscopy

To investigate the most active extract on its target cells, the extract at the 4× MIC level was incubated with the test microorganism for 24 h and was prefixed with 2.5% glutaraldehyde solution for 2 h. After that, cells were harvested by centrifugation and washed with 0.1 M phosphate buffer solution (pH 7.0), then post fixed with 1% osmium tetroxide. The sample was serially dehydrated with 50%, 70%, 80%, 90%, and 100% ethanol. Cells were dried by using a critical point dryer (Polarum, CP07501). The thin film of cells was smeared on a silver stub and observed under a scanning electron microscope (model JSM-5800 LV, type LV; JEOL Ltd., Japan) at the Scientific Equipment Center, Prince of Songkla University.

Alpha-glucosidase inhibition assay

Crude extracts were prepared at a concentration range of 0.5–0.1 mg/mL and subjected to an alpha-glucosidase inhibitory assay by the colorimetric method in a sterile 96-well plate according to Indrianingsih and Tachibana (2017) with some modifications. Fifty microliters of the extract, 25 μL of 0.3 unit/mL alpha-glucosidase solution, and 25 mL of potassium phosphate buffer (pH 6.8) were added to each well in triplicate for each crude extract. The plates were incubated at 37 °C for 10 min. After incubation, 25 μL of 10 mM 4-nitophenyl-alpha-D-glucopyranoside (*p*NPG) solution was added and incubated at 37 °C for 30 min. Then, 100 μL of 100 mM sodium bicarbonate solution (Na_2CO_3) was added to stop the reaction. The release of *p*-nitrophenol from *p*NPG was measured with a microplate spectrophotometer at 405 nm. Acarbose was used as the positive control. The results were expressed as the percentage inhibition by using the following formula: inhibitory activity (%) = $(1 - A_e / A_c) \times 100$, where A_e is the absorbance of the extract and A_c is the absorbance of the control. IC_{50} values were expressed and calculated using the GraphPad Prism program (GraphPad Software, Inc., USA)

In vitro cytotoxicity assay

The human lung cancer A549 cells were obtained from the Department of Optics and Mechatronics Engineering, Pusan National University, South Korea. They were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic solution (penicillin and streptomycin) in tissue culture flasks. The flasks were incubated at 37 °C in a humidified 5% CO_2 incubator for 2 days. The cells were suspended in the culture medium. Cell suspensions (5×10^4 cells) were mixed with extracts at a final concentration range of 200–6.25 μg/mL in 96-well tissue culture plates and incubated at 37 °C in a humidified 5% CO_2 incubator for 1 day. The visible of cell number was determined by the MTT method as in a previous study (Supaphon et al. 2018). Etoposide was used as the positive control.

GC-MS analysis

The chemical composition of broth extract (BE) was investigated by using the GC-MS analysis used by Supaphon et al. (2018) with some modifications. Briefly, the extract was suspended with methanol at 100 mg/mL and extract analysis was carried out with the GC system as follows: a VF-WAXms capillary column (30 m in length × 250 μm in diameter × 0. 25 μm in film thickness). Helium was used as the carrier gas at a flow rate of 1 mL/min, and the temperature program was 50–320 °C at 10 °C/min and maintained at 320 °C for 10 min. Mass spectrometry was conducted by electron ionization (70 eV) as follows: electron ion source at 230 °C and a mass range of 35–500 m/z. The result was compared with the Wiley Spectral Library. Each chemical component was expressed as the percentage of the peak area in the chromatogram.

Results

Fungal identification

Nigrospora was selected and identified based on their morphology. Their young hyphae are hyaline and septate and become brown in time. The color of the colony is white initially and then becomes gray or dark brown from both front and reverse. The conidiogenous cells on the conidiophores are inflated, swollen, and ampulliform in shape bearing a single black conidium (14–20 μm in diameter)

at their apex. In addition, ITS rDNA sequence analyses were used to classify *Nigrospora* sp. The phylogenetic trees were constructed by MP and ML analyses. Sequence similarity to sequences retrieved from GenBank databases was determined. Subsequently, BLAST search results indicated that the ITS rDNA sequences belong to the members of class Sordariomycetes, order Xylariales, and family Apiosporaceae. The generated phylogenetic alignment consisted of 43 taxa, with *Amphisphaeria sorbi* (MFLUCC 13-0721), *Phlogicylindrium eucalyptorum* (CBS111689), and *Phlogicylindrium uniforme* (CBS131312) as the outgroup.

The dataset constituted of 555 total characters, in which 405 characters were constant, 106 characters were parsimony informative, and 44 variable characters were parsimony uninformative. The best tree inferred a tree length of 254 steps [consistency index (CI) = 0.728, retention index (RI) = 0.861, relative consistency (RC) index = 0.627, and homoplasy index (HI) = 0.272]. One of the five MPTs presented in Fig. 1 had the best topology determined by the K-H test (Kishino and Hasegawa

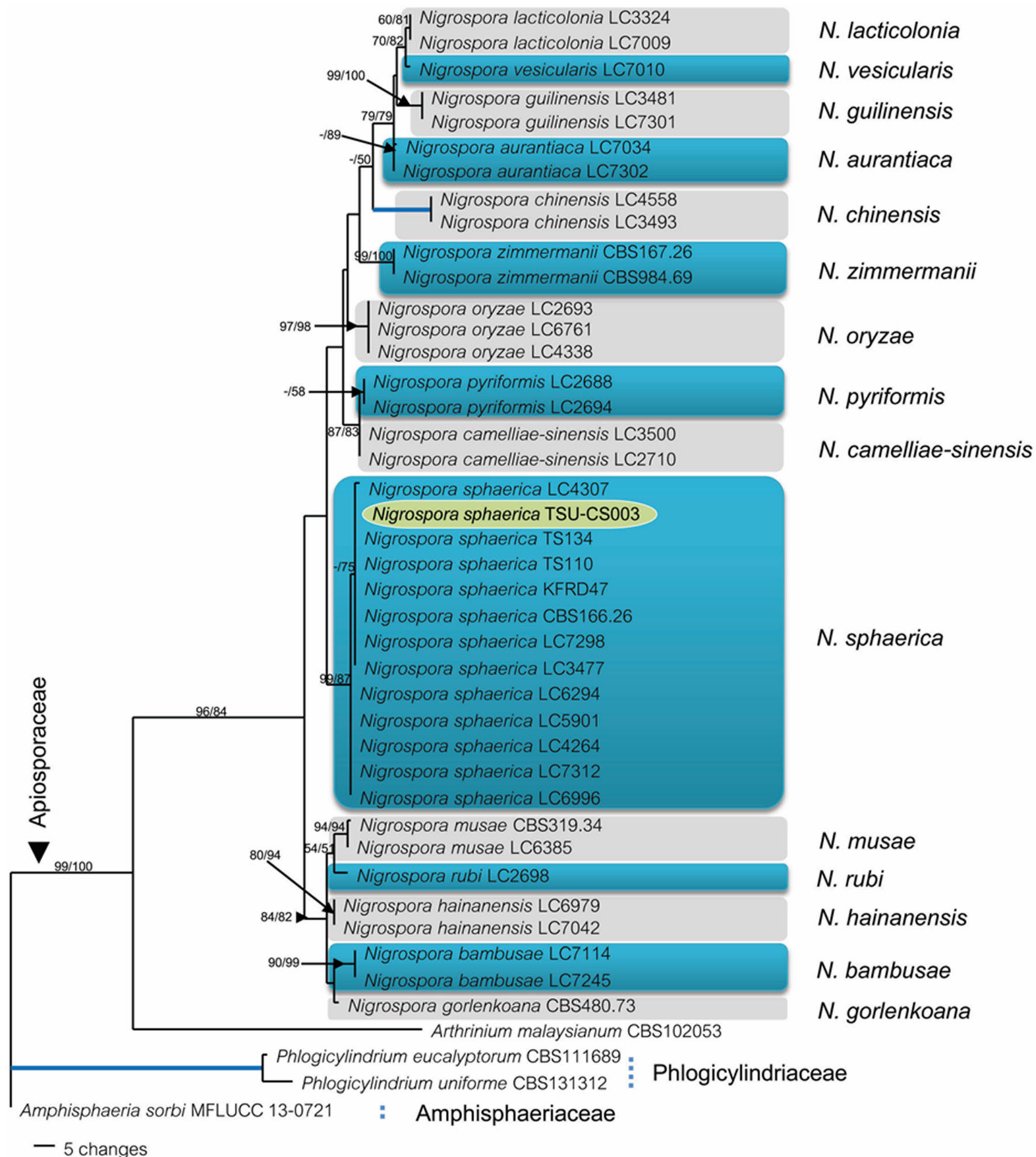

Fig. 1 One of four most parsimonious trees using the ITS rDNA gene with *Amphisphaeria sorbi* (MFLUCC 13-0721), *Phlogicylindrium eucalyptorum* (CBS111689), and *P. uniforme* (CBS131312) as the outgroup. The best phylogeny was performed by maximum parsimony analyses. Maximum parsimony bootstrap values (BSMP, left) and maximum likelihood bootstrap values (BSML, right) equal or greater than 50% are shown above each branch. The blue line shows strong support by all bootstrap values (100%)

1989). The maximum likelihood tree illustrated a similar topology to the MP tree (data not shown).

The phylogeny evidently demonstrated that our strain (TSU-CS003) was assigned as the genus *Nigrospora* with strong statistic support as shown in Fig. 1. Especially, the TSU-CS0003 sequence grouped together with *Nigrospora sphaerica* with high support (99% BSMP, 87% BSML). Thus, the isolates should be identified as *N. sphaerica*. Moreover, the sequences analyzed in this study were deposited at the GenBank databases. The alignment result was submitted in TreeBASE (submission number: 24396). The fungal isolates (culture collection number: TISTR3654) have been deposited at the culture collection of Thailand Institute of Scientific and Technological Research (TISTR), Thailand.

Antimicrobial activity

The colorimetric broth microdilution method was used to evaluate the MIC of endophytic fungal extracts against ten pathogenic microorganisms. All extracts exhibited antimicrobial activity against all test pathogenic microorganisms, except Gram-negative bacteria, with average MICs that ranged from 4 to 128 μg/mL and MFCs that ranged from 16 to 128 μg/mL (Table 1). The BE gave the highest activity against *T. marneffei* with an MIC and MFC value of 4 μg/mL and 16 μg/mL, respectively.

Furthermore, the antifungal activity of the most active extracts on their target cells was investigated by SEM. The results confirmed that this extract has the ability to destroy the cell wall of *T. marneffei*. Scanning electron micrographs of *T. marneffei* after treatment with BE at fourfold the MIC (16 μg/mL) for 24 h were compared with those of amphotericin B (positive control) and DMSO (negative control) and are shown in Fig. 2. The cell surface after treatment with the antifungal agent and extract was broken, collapsed, deformed, and withered, while mycelia after treatment with DMSO had a normal cell surface without any damage or broken cells.

In vitro alpha-glucosidase inhibitory assay

In this study, the extracts derived from the endophytic fungus *N. sphaerica* were evaluated for their ability to inhibit the enzyme alpha-glucosidase by colorimetric assay. The extracts (CE and CH) showed alpha-glucosidase inhibitory activity lower than 50% inhibition with inhibitory activity of 31% and 19%, respectively, whereas the BE gave the best alpha-glucosidase inhibitory activity of more than 50% inhibition. Thus, this extract was evaluated for IC_{50} value. Acarbose was used as the positive control, which showed an enzyme inhibitory activity with an IC_{50} value of 4.20 μg/mL, whereas the BE exhibited an inhibitory activity with an IC_{50} value of 17.25 μg/mL (Table 2). The endophytic extract had a higher IC_{50} than acarbose. This result might be due to the chemical components in the crude extract.

In vitro cytotoxicity assay

The active compound (BE) displayed potential in vitro cytotoxicity against the A549 human cancer cell lines. It yielded high cytotoxicity demonstrated by inhibition of cell proliferation in a concentration-dependent manner. In addition, the IC_{50} values of BE and etoposide were 22.41 ± 0.76 μg/mL and 37.57 ± 1.03 μg/mL, respectively. From the IC_{50} values, the BE was found to

Table 1 Antimicrobial activity of extracts obtained from *N. sphaerica* at concentrations ranging from 128 to 0.25 μg/mL

Microorganisms	MIC/MBC or MFC (μg/mL)						
	BE	CE	CH	Vancomycin	Gentamicin	Clotrimazole	Amphotericin B
Bacteria							
Staphylococcus aureus ATCC25923	16/128	64/128	128/128	0.5/1	–	–	–
Methicillin-resistant *S. aureus* (MRSA)	32/128	128/128	128/128	1/2	–	–	–
Escherichia coli ATCC25922	–	–	–	0.25/1	–	–	–
Pseudomonas aeruginosa ATCC27853	–	–	–	0.25/1	–	–	–
Yeast							
Candida albicans ATCC90028	62/128	128/128	64/128	–	–	–	0.25/1
C. albicans NCPF3153	64/128	128/128	128/128	–	–	–	0.125/1
Cryptococcus neoformans ATCC90112	64/128	128/128	128/128	–	–	–	0.25/1
C. neoformans ATCC90113	128/128	128/128	128/128	–	–	–	0.25/1
Filamentous fungi							
Microsporum gypseum clinical isolate	–	–	–	–	–	1/32	–
Talaromyces marneffei clinical isolate	4/16	128/128	–	–	–	–	0.25/2

BE fermentation broth extracted with ethyl acetate, *CE* mycelia extracted with ethyl acetate, *CH* mycelia extracted with hexane

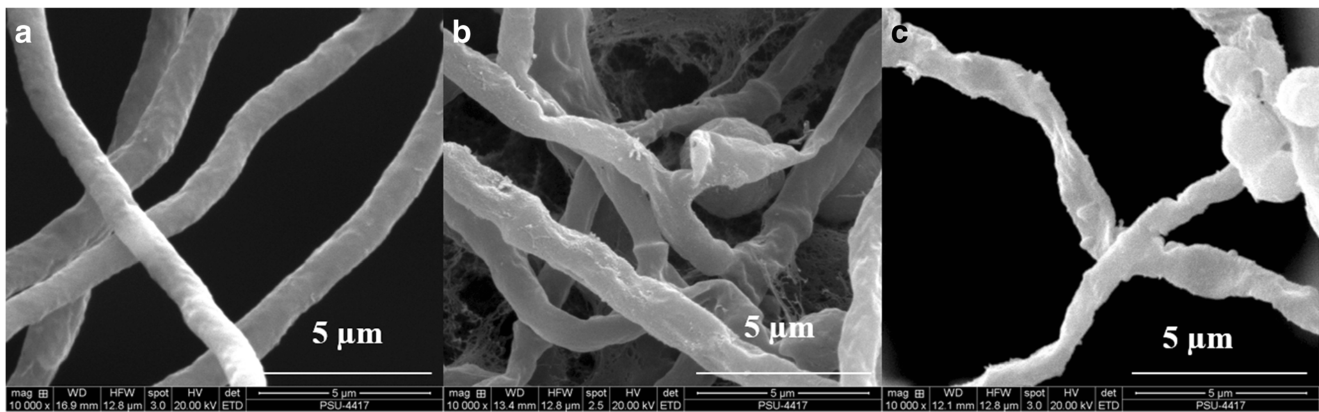

Fig. 2 Scanning electron micrograph of *T. marneffei* after treatment with 1% DMSO (**a**), 4× MIC amphotericin B at 1 μg/mL (**b**), and BE 4× MIC at 16 μg/mL (**c**)

have strong antiproliferative activity against the A549 human cancer cells lines. It showed a significantly higher cytotoxicity than etoposide, the positive control (Fig. 3).

GC-MS analysis

The GC-MS analysis was used to identify active metabolites in the crude extract. The identification of this BE was compared with the Wiley Library databases, as well as by retention index and determination of each peak area in percentage from the chromatograms. The compounds in the ethyl acetate fraction obtained from *N. sphaerica* had a match score of over 90% in the library as shown in Table 3. The three main compounds present in the ethyl acetate extract were 5-pentyldihydrofuran-2(3*H*)-one (56.38%), (Z)-methyl 4-(isobutyryloxy)but-3-enoate (27.62%), and 2-phenylacetic acid (4.08%). The total minor constituents were present at 11.92%.

Discussion

Antimicrobial activity

At present, the treatment of diseases by medicinal drugs and the control of their side effects are very important. Researchers aim to reduce the side effect of drugs to an acceptable level. This problem is a huge global challenge. In the past decades, researchers have sought new sources of novel metabolites from many substrates, especially from natural products. Plants are an important source of active agents to combat this problem and have been used as alternative sources for treatment for over 100 years. However, many plant metabolites constitute variations of known chemical compounds, while fungal endophytes have attracted greater attention due to their ability to produce several active secondary metabolites. Thus, endophytic fungi are being extensively examined for discovery of new pharmaceutical agents. The results of the present study showed that the BE from *N. sphaerica* gave the best antifungal activity against *T. marneffei* with MIC and MFC values of 4 μg/mL and 16 μg/mL, respectively, a maximum alpha-glucosidase inhibitory activity with an IC_{50} value of 17.25 μg/mL and high cytotoxicity to A549 human lung cancer cell lines with an IC_{50} value of 22.41 μg/mL. For antimicrobial activity, crude extracts from this fungus expressed a good ability against *T. marneffei*, which is a pathogenic fungus that causes systemic fungal infection and opportunistic infections in human immunodeficiency virus (HIV)–infected patients. This disease is referred to as talaromycosis. Many antifungal agents were used for treatment, such as ketoconazole, miconazole, and amphotericin B. However, they present many side effects to the patients. They may also cause liver damage and kidney problems (Foster et al. 1981; Bensom and Nahata 1988). Thus, the evaluation of antimicrobial potential of active secondary metabolites from natural is needed. Overall sensitivity of *T. marneffei* was obtained from previous studies. Jeenkeawpieam et al. (2012) investigated the antimicrobial

Table 2 Alpha-glucosidase inhibition of ethyl acetate partition extract from fermentation broth (BE) obtained from *N. sphaerica*

Sample	% inhibitory concentration (μg/mL)								IC_{50} (μg/mL)
	250	125	62.50	31.25	15.63	7.81	3.90	1.95	
Extract	91.70 ± 2.40	82.75 ± 1.50	75.67 ± 1.50	69.71 ± 1.16	65.23 ± 0.52	45.56 ± 0.14	40.22 ± 0.11	38.10 ± 0.04	17.25
Acarbose	98.92 ± 3.55	97.25 ± 2.81	96.20 ± 2.11	85.35 ± 1.95	67.50 ± 1.11	65.20 ± 0.52	55.75 ± 0.12	49.20 ± 0.05	4.20

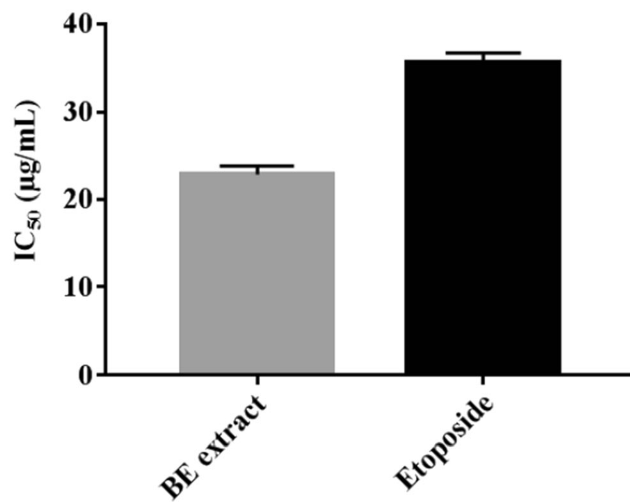

Fig. 3 Cytotoxic effect of BE from the fermentation broth of *Nigrospora sphaerica* TSU-CS003 on the A549 human lung cancer cell lines as determined by the MTT assay for 72 h

activity of crude extracts from endophytic fungi which were isolated from *Rhodomyrtus tomentosa*. They found that many strains of endophytic fungi (*Guignardia mangiferae*, *Phomopsis* sp., *Phomopsis pyllanthicola*, *Diaporthe* sp., *Diaporthe phaseolorum*, *Colletotrichum gloeosporioides*, *Colletotrichum* sp.) yielded good antifungal activity against *T. marneffei* with MICs that ranged from 2 to 200 µg/mL, especially from endophytic fungi belonging to the genus *Colletotrichum* which showed the lowest MIC value of 2 µg/mL. In addition, the endophytic fungus *Fusarium* sp. obtained from seagrass (*Enhalus acoroides*) exhibited antifungal activity against *T. marneffei* with an MIC value of 200 µg/mL (Supaphon et al. 2014). Our findings on the potential of endophytic fungi isolated from *H. annuus* are supported by these results. However, the difference in the sensitivity among bacteria, yeasts, and filamentous fungi may be due to the variation of their cell components or target sites for active metabolites (Russell 2003).

Alpha-glucosidase activity

The inhibition of alpha-glucosidase is useful for diabetes treatment. If the inhibitors can delay digestion of carbohydrate, they can control and prevent high levels of blood glucose. Acarbose is an antidiabetic drug that inhibits alpha-glucosidase, but it has gastrointestinal side effects (Rosak and Mertes 2012). Hence, the extracts of *N. sphaerica* were evaluated for inhibition of alpha-glucosidase. The BE showed the highest inhibition compared to the other extracts (CE and CH extracts). Alpha-glucosidase inhibitory activity from endophytic fungi has previously been reported. Indrianingsih and Tachibana (2017) found that the extract (8-hydroxy-6,7-dimethylisocoumarine) from the endophytic fungus *Xylariaceae* sp. had alpha-glucosidase inhibitory activity with an IC_{50} value of 41.75 µg/mL. Singh and Kaur (2015) extracted the endophytic fungus (*Aspergillus awamori*) with ethyl acetate, chloroform, butanol, and hexane and tested for their anti-alpha glucosidase activity. They reported that the ethyl acetate extract significantly inhibited alpha-glucosidase with an IC_{50} value of 5.625 µg/mL. In addition, there are many reports on the potential of extracts from endophytic fungi (*Nectria* sp., *Trichoderma* sp., *Zasmidium* sp., *Aspergillus* sp., *Penicillium* sp., and *Cladosporium* sp.) that exhibited strong alpha-glucosidase inhibitory activity (Cui et al. 2016; El-Hady et al. 2016; Murugan et al. 2017; Zhang et al. 2017; Popli et al. 2018). However, reports of the endophytic fungus *Nigrospora* sp. producing alpha-glucosidase inhibitors are rare. Zhu et al. (2017) found two new compounds (nigrosporamide A and derivative, 4-prenyloxyclavatol) from *N. sphaerica*, which was isolated from *Oxya chinensis* that exhibited strong inhibitory activity towards alpha-glucosidase. These previous findings confirm that endophytic fungi are potential sources of alpha-glucosidase inhibitors.

Cytotoxicity and GC-MS analysis

The cytotoxicity of BE on human lung cancer cell lines was also examined. It was found that this extract was more cytotoxic on the tested lung cancer cell lines than the positive control (etoposide). This finding agrees with previous reports. Ayob et al. (2017) studied the cytotoxicity of an extract derived from *N. sphaerica* on the MDA-MB 231 breast cancer cell line. They found that the extract from this fungus has high cytotoxicity with an IC_{50} value of 0.868 µg/mL. Lu et al. (2012) reported that extracts from many strains of endophytic fungi (*Acremonium* sp., *Trichoderma* sp., and *Chaetomium* sp.) displayed cytotoxicity against the A549 human lung cancer cells with IC_{50} that ranged from 16.22 to 89.15 µg/mL. Goutam et al. (2017) found that the endophytic fungus (*Aspergillus terreus*) demonstrated cytotoxicity against the A549 human lung cancer cell lines with an IC_{50} value of 121.9 µg/mL.

However, this is the first report of the endophytic fungus *N. sphaerica* from *H. annuus*. The major components of BE

Table 3 GC-MS analysis of ethyl acetate partition extract from fermentation broth (BE) of *N. sphaerica*

No.	Compound	Match factor	% content
1	5-Pentyldihydrofuran-2(3*H*)-one	92.7	56.38
2	(Z)-Methyl 4-(isobutyryloxy)but-3-enoate	94.8	27.62
3	2-Phenylacetic acid	99.6	4.08

are 5-pentyldihydrofuran-2(3*H*)-one, (Z)-methyl 4-(isobutyryloxy)but-3-enoate, and 2-phenylacetic acid. Some of these major constituents have been previously reported as pharmacological agents. 2-Phenylacetic acid, a phytoconstituent of *Lantana camara* methanolic leaf extract and a fungus (*Glomerella cingulata*), is known to possess strong antimicrobial and antityrosinase activities (Hirota et al. 1993; Zhu et al. 2011; Fayaz et al. 2017). However, the biological activities of other compounds [5-pentyldihydrofuran-2(3*H*)-one and (Z)-methyl 4-(isobutyryloxy)but-3-enoate] have not been reported. Thus, the strong biological activities in this study exhibited by the BE could be due to the presence of 2-phenylacetic acid or the synergistic effect of the compounds. *Nigrospora* spp. produce various bioactive secondary metabolites, including nigrosporin, pyrone derivatives, tyrosol, taxol, and alkaloids (Arumugam et al. 2014). However, many secondary metabolites contain constituents that could be toxic. In addition, over 1000 secondary metabolites of fungal origin have now been chemically characterized. They have been difference in chemical compositions and often are species specific. However, very few are known that these natural compounds are extremely toxic to human and have adverse effects. Some of these fungi produce potent toxic metabolites (Yuan et al. 2016).

The cytotoxicity profile and adverse actions of active metabolites should be tested and explored. In addition, isolation, characterization, and evaluation of each compound are important for further studies.

In conclusions, this research clearly showed that the endophytic fungus *N. sphaerica* demonstrated a significantly broad spectrum of alpha-glucosidase inhibition, antimicrobial, and cytotoxic inhibitory activities. The BE gave the highest antifungal activity against *T. marneffei* with MIC/MFC values of 4 μg/mL and 16 μg/mL, respectively, compared with amphotericin B as a standard antifungal agent. Furthermore, it showed the ability to inhibit alpha-glucosidase with an IC_{50} value of 17.25 μg/mL and had cytotoxicity against the A549 human lung cancer cell line. It should be considered for further studies involving investigation of other biological activities and further applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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