Vol. 13(27), pp. 464-473, September, 2019
DOI: 10.5897/AJMR2019.9164
Article Number: 2CC499E61808
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

Antimicrobial and antioxidant activities of endophytic fungi extracts isolated from *Carissa carandas*

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Received 2 July, 2019; Accepted 22 August, 2019.

This is the first report of endophytic fungi derived from *Carissa carandas* producing bioactive compounds in Thailand. The aims of this research were to evaluate the antimicrobial and antioxidant activities of extracts from endophytic fungi, identify the potential fungal isolates by phylogenetic analysis and analyze the composition of the potential crude extract by gas chromatography-mass spectrometry (GC-MS). The endophytic fungus *Nigrospora guilinensis* TSU-EFHA009 produced the most active extracts. Broth ethyl acetate extract (BE) had the strongest activity against *Cryptococcus neoformans* at a minimum inhibitory concentration (MIC) of 4 µg/mL and a minimum fungicidal concentration of 8 µg/mL. Moreover, the antimicrobial activity was confirmed using scanning electron microscopy. The target cells were morphologically damaged. In addition, this active extract had the highest antioxidant activity with an inhibitory concentration (IC_{50}) value of 0.03 mg/mL. The total phenolic content of the target extract was detected by using the colorimetric method. This extract contained a total phenolic content of 41.20±0.40 mg GAE/g of the extract. The results indicated that the endophytic fungi from *C. carandas* are good sources of antimicrobial and antioxidant substances.

Key words: Antimicrobial activity, antioxidant activity, endophytic fungi, active metabolites.

INTRODUCTION

Nowadays, drug resistant microorganisms and free radical agents are gaining more attention. They lead to infectious diseases in humans and to various other diseases (cancer, asthma and cardiovascular disease). Infectious disease and free radicals are important issues found in all regions of the world (Racek et al., 2001; Hubalek, 2003; Lobo et al., 2010; Lindahl and Grace, 2015). Increasing attention has been paid to natural products, especially those from fungal endophytes. It is well documented that fungal endophytes are a good source of bioactive natural compounds that are effective, have low toxicity, and cause a minimum environmental impact (Jalgaonwala et al., 2011; Nisa et al., 2015). There are several definitions of endophytic fungi; one of the recent definitions is that it is fungus which colonizes host plant tissue without visible symptoms (Jia et al., 2016; Gouda et al., 2016). Recent studies have reported that the functional potential of fungal endophytes is derived from many terrestrial plant species (Joseph and Priya, 2011). In some cases, novel compounds from fungal

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endophytes showed strong antimicrobial activities against pathogenic microorganisms such as antibacterial, antifungal and anti-parasitic, and had strong antioxidant activities (Ascencio et al., 2014; Brissow et al., 2017; Raunsai et al., 2018). Active compounds (trichodermin and volatile compound) isolated from the fungal endophyte *Trichoderma* species had a strong activity against pathogenic bacteria and pathogenic fungi (Leylaie and Zafari, 2018). Fusaripeptide A isolated from the fungal endophyte *Fusarium* species which is isolated from roots of *Mentha longifolia* L. showed strong activity against *Plasmodium falciparum* (antimalarial) with an IC_{50} value of 0.34 µM, and against *Candida albicans, Candida glabrata, Candida krusei* and *Aspergillus fumigates* (antifungal) with IC_{50} values of 0.11, 0.24, 0.19 and 0.14 µM, respectively (Ibrahim et al., 2015). Furthermore, fungal endophytes can produce active antioxidant compounds (Huang et al., 2007; Khiralla et al., 2015). Bioactive natural products from fungal endophytes, in particular from *Carissa carandas*, have been rarely studied (Yadav et al., 2014; Tenguria and Firodiya, 2015, 2016). Previous studies on fungal endophytes which were isolated from *C. carandas* have been focused on biodiversity, distribution and cytotoxicity of endophytic extracts (Tenguria et al., 2012; Tenguria and Firodiya, 2015).

Carissa is a medicinal plant belonging to the Apocynaceae family comprised 20 to 30 species and is found in many parts of Asia, Africa and Australia. The common species of this genus are *C. carandas, Carissa macrocarpa, Carissa grandiflora, Carissa edulis, Carissa spinarum, Carissa lanceolata, Carissa opaca, Carissa congesta* and *Carissa bispinosa*. Many parts of this plant (stem, root, bark, fruit and seed) have been used in traditional medicine for thousands of years. Its fruit are used as a treatment for various ailments such as liver dysfunction, fever, digestion system problems and diarrheas (Arif et al., 2016). Recently, Toobpeng et al. (2017) reported the antibacterial activity of fruit extracts against *Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Staphyloccus aureus*, methicillin-resistant *S. aureus, Acinetobacter baumannii*, *Enterococcus faecalis,* and *Schleichera oleosa*. However, the biological activities, especially, antimicrobial and antioxidant activities of fungal endophytes from *C. carandas* have not been studied.

Thus, this study aimed to isolate endophytic fungi from healthy fresh leaves of *C. carandas* and to screen these microorganisms for their ability to produce antimicrobial metabolites against human pathogens and antioxidant substances that inhibit or delay the oxidation of biologically relevant molecules.

MATERIALS AND METHODS

Sample collection and fungal isolation

Healthy leaf samples of *C. carandas* were randomly collected during January to March 2018 from Trang, Thailand (7° 33' 22.79" N; 99° 36' 41.08" E). Samples were surface-sterilized with 10% ethanol (5 min), 3% sodium hypochlorite (15 s), and 10% ethanol (5 min), and rinsed with distilled water and dried on sterile tissue paper. Each of the leaf parts was cut into five segments (0.5 cm^2) and put onto Potato Dextrose Agar (PDA) provided with antibiotics (50 mg/L penicillin and streptomycin). Plates were incubated at room temperature for 4 weeks. Fungal isolates were subcultured in PDA without antibiotics until they were pure cultures. All fungal isolates were identified and selected for further study based on their morphological characteristics after the incubation period. Each pure fungal isolate on PDA was cut into small pieces and maintained in 20% glycerol at -80°C.

Fermentations and extractions

Fermentation and extraction methods were conducted according to Supaphon et al. (2010, 2013) with some modifications. Six agar plugs (1 cm^2) of fungal mycelium were inoculated in 500 mL Erlenmeyer flasks containing 250 mL potato dextrose broth (PDB) and were incubated for 3 weeks at room temperature for production of metabolites. The culture broth was filtered to separate the filtrate and mycelia. The filtrate was extracted with an equal volume of ethyl acetate (EtOAc) in a separating funnel two times. The combined EtOAc extracts were evaporated to dryness under reduced pressure at 45°C using a rotary vacuum evaporator to obtain the broth ethyl acetate extract (BE extract). The fungal mycelia were soaked in 500 mL of methanol (MeOH) for 3 days. The aqueous MeOH layer was concentrated using rotary evaporator to give the aqueous layer. The aqueous layer was extracted with an equal volume of hexane two times, followed by EtOAc twice. The combined EtOAc and hexane extracts were evaporated to dryness under reduced pressure at 45°C using a rotary vacuum evaporator to obtain the cell ethyl acetate and cell hexane extracts (CE and CH extracts). The stock extracts were kept in vials at 4°C. Working extracts were kept in vials at room temperature until used.

Antimicrobial assay

The dried extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions at 100 mg/mL. All the extracts at a final concentration of 200 µg/mL were screened for their antimicrobial activity against ten pathogenic microorganisms, including *S. aureus* ATCC25923, a clinical isolate of methicillin-resistant *S. aureus* (MRSA) SK1, *E. coli* ATCC25922, and *P. aeruginosa* ATCC27853, *C. albicans* ATCC90028, *C. albicans* NCPF3153, *Cryptococcus neoformans* ATCC90112, *C. neoformans* ATCC90113, a clinical isolate of *Microsporum gypseum* and *Talaromyces marneffei* by the colorimetric microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) with slight modifications (CLSI 2008, 2012). Microtiter plates were incubated at 35°C for 15 h, then 30 µL of resazurin solution (0.18%) was added to each well and incubated for 3 h under the same conditions (Supaphon et al., 2018). After incubation, the results were recorded as positive (blue color indicated growth inhibition) and negative (pink color indicated microbial growth). After that, the active extracts from the screening test were determined by the same method for the minimum inhibitory concentrations (MICs) at a concentration range of 0.25 to 128 µg/mL. The lowest concentration of extract that inhibited growth was recorded as MIC. The concentration of extract at MIC and more than MIC were determined by streaking onto nutrient agar (NA) plates for bacteria, Sabouraud's dextrose agar (SDA) plates for yeasts and PDA plates for filamentous fungi and incubated under appropriate conditions. The lowest concentration of extract that showed no growth was recorded as the minimum bactericidal

concentration (MBC) for yeast and the minimum fungicidal concentration (MFC) for filamentous fungi. Commercial antibiotics were used as standard agents for positive inhibitory controls (vancomycin for Gram-positive bacteria, gentamicin for Gram-negative bacteria, amphotericin for yeasts and *T. marneffei* and miconazole for *M. gypseum*).

Scanning electron microscopy analysis

The effect of the most active extract on cell morphology was determined using scanning electron microscopy (SEM). The sample was prepared according to previous studies with slight modifications (Supaphon et al., 2018). Briefly, cell suspension (10^8 CFU/mL) was treated with four times MIC concentration of the active extract and incubated for 24 h. For the controls, antibiotics and DMSO were used as positive and negative controls, respectively. The treatments were fixed with 2.5% glutaraldehyde in a phosphate buffer solution for 1 h and washed three times with PBS, pH 7.2. Each treatment was serially dehydrated with 50, 70, 80, 90 and 100% ethanol. Then, cell samples were dried in a lyophilizer, smeared on a silver stub, mounted with gold and observed using SEM at the Scientific Equipment Center, Prince of Songkla University.

Gas chromatography-mass spectrometry analysis (GC-MS)

GC-MS was used to determine the active extracts. This experiment was performed according to the previous study with some modifications (Supaphon et al., 2018). The separation and identification of the compounds (extract from *Nigrospora guilinensis*) used the HP5MS capillary column (30 m × 0.25 mm ×0.5 µm). The temperature program was performed as follows: initial temperature of 50°C (2 min), raised to 160°C at the rate of 8°C/min (5 min), then raised to 270°C at the rate of 8°C/min (8 min), Helium was used as the carrier gas at the rate of 10 mL/min. The fragmentation of the MS range from 40 to 1000 m/z was conducted by electronic impact mode (ionization energy, 70 eV, 300°C) and scanned at the rate of 3.0 scans/s. GC-MS was analyzed for 50 min.

Determination of antioxidant activity (DPPH) assay

1, 1- diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity of endophytic fungi extracts was evaluated according to the previous report of Yadav et al. (2014) with slight modifications. Briefly, the DPPH solution was prepared by dissolving 2.4 mg DPPH in 100 mL methanol, and the stock solution was kept at -20°C until used. The extract solution at a concentration of 10 mg/mL (50 µL) was added to 50 µL of 1 mM DPPH solution in 96-well microtiter plates. The mixture was shaken and stored at room temperature for 30 min in the dark, and then the absorbance was recorded at 515 nm using a spectrophotometer. For the control, the absorbance of ascorbic acid solution was used as a standard for making the calibration curve using 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL concentrations of the standard. All determinations were performed in triplicate. The percent scavenging activity was calculated using the formula: $[A_0-A_s/A_0] \times 100$, where A_0 and A_s represent the absorbance values of the control and extract, respectively. The active extracts that provided ≥50% scavenging activity were identified by interpolation from linear regression analysis.

Determination of total phenolics contents

The total phenolic content of the broth ethyl acetate extracts was measured as described by Yadav et al. (2014) with some modification. The extracts were prepared and diluted from stock solutions (1 mg/mL). 100 µL of fungi extract was diluted with 7 mL water and then was mixed with 500 µL of Folin-Ciocalte. The mixture solution was incubated at room temperature for 4 min. Then 1.5 mL of sodium carbonate (7.5%) was added and kept in the dark at room temperature for 2 h. The results were recorded by measuring the absorbance at 765 nm using a spectrophotometer (Thermo Scientific). Gallic acid was used as positive control, and a standard curve at the concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mg/mL in 70% of methanol was constructed. The results were reported as mg gallic acid equivalent dry weight (GAE/g of extract). Each treatment was performed in triplicate.

Molecular traits

The most active isolate as determined by the antimicrobial and antioxidant activity assays was further identified by molecular methods based on ITS sequence analyses. Extraction of fungal mycelium used the modified CTAB method from O'Donnell et al. (1997). Afterwards, the qualities of genomic DNA were estimated with 1% agarose gel electrophoresis in 1% TAE buffer. Internal transcribed spacer (ITS) rDNA was amplified by PCR with the primer pairs ITS5/ITS4 (White et al., 1990). The PCR reactions used Taq DNA polymerase (Thermo Scientific) following the manufacturer's instructions and were performed in a T100TM Thermal cycler (BIO-RAD laboratories, Inc). Furthermore, the amplification conditions of ITS rDNA were performed as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min, with a final extension period of 72°C for 10 min. The PCR products were checked on 1% agarose electrophoresis gels and stained with RedSafe DNA stain (20,000X). After that, PCR products were purified by Macrogen Inc. in South Korea for direct DNA sequencing.

Phylogenetic analysis

Nucleotide sequences in this study were compared with the related sequences from GenBank (http://www.ncbi nlm.nih.gov) and 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. Maximum parsimony analyses were performed in PAUP*4.0b10 (Swofford, 2002). The most parsimonious trees (MPTs) results were 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 supports of the clades were 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). Moreover, the sequences analyzed in this study were deposited in the GenBank databases and are shown in Table 4. The alignment result was submitted to TreeBASE (submission number: 24397).

RESULTS AND DISCUSSION

Fungal isolation

One-hundred and nine fungal isolates from 900 segments

of *C. carandas* were identified based on their morphology as *Penicillium,* 20 isolates; *Cladosporium,* 13 isolates; *Fusarium*, 6 isolates; *Curvularia*, 5 isolates; and *Nigrospora* 3 isolates. The remaining fungal isolates (62 isolates) did not produce any reproductive structure and were classified as unidentified endophytic fungi. All endophytic fungal isolates were grouped into 36 morphotypes based on their morphology. Representative isolates were selected from each group for antimicrobial and antioxidant activity tests.

Antimicrobial assay

The preliminary screening of crude extracts (108 extracts) from selected endophytic fungi (36 isolates) revealed the presence of bioactive compounds. The antimicrobial activity was determined by using the colorimetric broth microdilution assay. The activities of crude extracts were evaluated at a concentration of 200 µg/mL. Fourteen extracts (BE = 6, CE = 5 and CH = 3) exhibited significant inhibition against at least one test microorganism. The MIC test showed potential antagonism against two strains of Gram-positive bacteria (*S. aureus* ATCC25923 and a clinical isolate of methicillin-resistant *S. aureus* (MRSA) SK1), four strains of yeasts (*C. albicans* ATCC90028, *C. albicans* NCPF3153, *C. neoformans* ATCC90112 and *C. neoformans* ATCC90113) and two strains of filamentous fungi (clinical isolate of *M. gypseum* and *T. marneffei*) at concentrations ranging from 4 to 128 µg/mL, but not against Gram-negative bacteria (*E. coli* ATCC25922 and *P. aeruginosa* ATCC27853). The BE from isolate TSU-EFHA009 inhibited the growth of *C. neoformans* ATCC90112 at the lowest concentration of 4 µg/mL and produced an MFC at the lowest concentration of 8 µg/mL; while this extract inhibited other test microorganisms at moderate to high concentrations ranging from 32 to 128 µg/mL shown in Table 1.

The SEM analysis

The cell surface morphology of *C. neoformans* ATCC90112 was observed after treatment with the most active extract (BE extract from TSU-EFHA009) using SEM. Cells after treatment with the extract at a concentration of 4X MIC (16 µg/mL) appeared to be shrunken, with broken and wrinkled cell surfaces, similar to cells treated with amphotericin B at a concentration of 1 µg/mL (positive control). Whereas, the morphology of cells treated with 1% DMSO (negative control) exhibited a normal cell surface without any damage (Figure 1). These SEM analyses indicated that the BE extract had effects on cell wall of *C. neoformans.*

Antioxidant activity and total phenolic content

Crude extracts of 36 fungal isolates were screened for their antioxidant properties (DPPH assay) in comparison with an antioxidant agent (ascorbic acid). From the DPPH radical scavenging activity results, three out of the 108 extracts showed strong antioxidant activity with 90% inhibition, while ascorbic acid gave a 95% inhibition. Among the active extracts (three extracts) had an excellent scavenging effect, especially the BE from TSU-EFHA009. The inhibitory concentration value (IC_{50}) of this extract was 0.03 mg/mL (Table 2).

Phylogenetic relationship of active fungal isolates

ITS rDNA sequences analyses were used to classify the selected endophytic fungi. The phylogenetic trees were performed by MP and ML analyses. The sequence similarity of sequences retrieved from GenBank databases was determined. Subsequently, BLAST search results of ITS rDNA sequences indicated that the isolates belonged to the class Sordariomycetes, order Xylariales, and family Apiosporaceae. The generated phylogenetic alignment consisted of 48 taxa (Table 4), with *Amphisphaeria sorbi* (MFLUCC 13-0721), *Phlogicylindrium eucalyptorum* (CBS111689) and *Phlogicylindrium uniforme* (CBS131312) as outgroup.

The dataset constituted 607 total characters; 385 characters were constant; 191 characters were parsimony informative and 31 variable characters were parsimony uninformative. The best tree inferred a length of 443 steps [consistency index (CI) = 0.693, retention index (RI) = 0.873, relative consistency index (RC) = 0.605, homoplasy index (HI) = 0.307]. One of the ten MPTs is as shown in Figure 2; the best topology was determined by the K-H test (Kishino and Hasegawa, 1989). The maximum likelihood tree illustrated a similar topology to the MP tree (data not shown).

The phylogenetic results demonstrated that our strain (TSU-EFHA009) was assigned to genus *Nigrospora* with strong statistic support as shown in Figure 2. It is grouped together with the *N. guilinensis* clade (LC3481 and LC7301), with strong support (65% BSMP and 64% BSML) and their numbers of nucleotide substitutions exhibited 507/515 = 98.5% similarity with eight substitutions. Hence, this strain should be classified taxonomically as *N. guilinensis*.

GC-MS analysis

The CE extract from isolate TSU-EFHA 009 was analyzed by GC-MS as shown in Table 3. In this study, retention indices were also compared to the published values. An agreement above 90%, of the spectra, was considered for identification of constituents. The CE could be divided into 10 components, the majority of which are 4-(cyclopentyloxy) cyclohex-2-en-1-yl acetate (21.89%), 5,7a-dimethyloctahydro-1-inden-3a-yl)(phenyl)methanone (12.37%) and 2-methylcyclohexanone (10.02%). Three

Table 1. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) of the active extracts from fungal endophytes at a final concentration range of 0.25 to 128 µg/mL.

Extract	Fungal code	Test microorganisms									
		MIC/MBC or MFC values (µg/mL)									
		Bacteria				Yeasts				Filamentous fungi	
		SA	MRSA	EC	PA	CA1	CA2	CN1	CN2	MG	TM
BE	TSU-EFHA 001	64/128	-	-	-	128/128	128/128	128/128	-	-	-
	TSU-EFHA 003	128/128	-	-	-	128/128	-	-	128/128	-	-
	TSU-EFHA 005	64/128	64/128	-	-	-	-	-	-	-	-
	TSU-EFHA 006	-	-	-	-	64/128	32/64	-	-	-	-
	TSU-EFHA 008	8/16	128/200	-	-	-	64/128	128/128	-	-	128/128
	TSU-EFHA 009	32/64	64/128	-	-	64/128	32/64	4/8	64/128	128/128	-
CE	TSU-EFHA 003	128/128	-	-	-	-	-	-	128/128	-	-
	TSU-EFHA 006	128/128	-	-	-	-	-	-	-	-	-
	TSU-EFHA 008	32/64	32/64	-	-	-	-	-	-	-	-
	TSU-EFHA 003	-	64/128	-	-	-	-	-	-	-	-
	TSU-EFHA 012	-	128/128	-	-	-	-	-	128/128	-	-
CH	TSU-EFHA 003	128/128	-	-	-	-	-	-	-	-	-
	TSU-EFHA 006	128/128	-	-	-	-	-	-	-	-	-
	TSU-EFHA 008	128/128	-	-	-	-	-	-	-	-	-
Control	Vancomycin	0.25/0.5	0.5/1.0	ND	ND	ND	ND	ND	ND	ND	ND
	Gentamicin	ND	ND	0.5/2	0.5/2	ND	ND	ND	ND	ND	ND
	Amphotericin B	ND	ND	ND	ND	0.125/0.5	0.125/0.5	0.25/1.0	0.25/1.0	ND	ND
	Crotimazole	ND	ND	ND	ND	ND	ND	ND	ND	2/4	1/2

CH, Hexane extract from fungal mycelium; BE, ethyl acetate extract from culture broth; CE, ethyl acetate extract from fungal mycelium. MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration. SA, *Staphylococcus aureus* ATCC25923; MRSA, methicillin-resistant *Staphylococcus aureus* SK1; EC, *Escherichia coli* ATCC25922; PA, *Pseudomonas aeruginosa* ATCC27853; CA1, *Candida albicans* ATCC90028; CA2, *Candida albicans* NCPF 3153; CN1, *Cryptococcus neoformans* ATCC90112 (flucytosine-sensitive); CN2, *Cryptococcus neoformans* ATCC90113 (flucytosine-resistant); MG, *Microsporum gypseum* clinical isolate; TM, *Talaromyces marneffei* clinical isolate.

main components were identified in the BE fraction and represented over 10% of the peak area. The remaining components were present at <10% of the peak area.

Many secondary metabolites are produced by the fungal endophytes. Thus, this study aimed to evaluate the antimicrobial and antioxidant activities of such fungal isolates. They may be a renewable source of novel bactericidal, fungicidal and antioxidant activities. The effects of the endophytic extracts in this study against tested pathogenic microorganisms were significant, except against Gram-negative bacteria. This might be because of the structure of these bacteria. There is an outer membrane that prevents a sufficient active agent

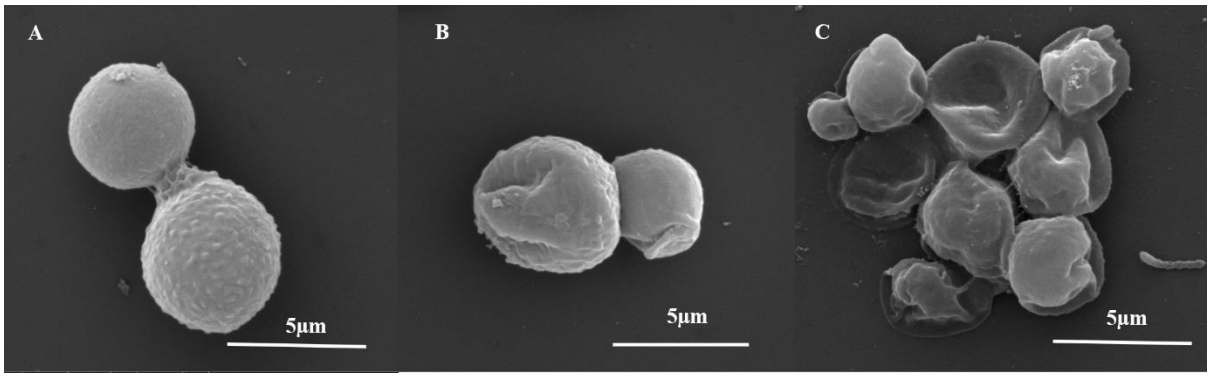

Figure 1. Scanning Electron Micrograph of *Cryptococcus neoformans* ATCC 90112. (A) Cell surface of *C. neoformans* after treatment with 1% DMSO. (B) Cell surface of *C. neoformans* after treatment with amphotericin B (1 µg/mL). (C) *C. neoformans* after treatment with active extract (Broth ethyl acetate extract from isolate TSU- EFHA 009) at 4X MIC value (16 µg/mL).

Table 2. DPPH scavenging activity and total phenolic content of the potential crude extracts.

Crude extract	DPPH scavenging activity (%)	IC_{50} values (mg/mL)	Total phenolic content (mg of GAE/g of extract)
TSU-EFHA 005	92	0.10	19.83±0.11
TSU-EFHA 009	95	0.03	41.20±0.40
TSU-EFHA 010	90	0.25	4.30±0.25
Ascorbic acid	98	0.001	-

effect (Beveridge, 1999). This is the first report that evaluated the antimicrobial and antioxidant properties of endophytic fungi from *C. carandas*, while other reports focused on the biological activities and compound identification from *C. carandas*.

Pawle and Singh (2014) isolated fungal endophytes (*Nigrospora* species) from the living fossil *Ginkgo biloba*. The ethyl acetate extracts from culture broth showed antimicrobial activity with MIC values of 2.5 mg/mL against *E. coli, Klebsiella* species, *S. aureus, C. albicans* and *Geotrichum* species, while the ethyl acetate extract from *N. guilinensis* TSU-EFHA009 in this study yielded high activity against *C. neoformans* with an MIC value of 4 µg/mL and MFC of 8 µg/mL. These results give credence that extracts from fungal endophytes show great antimicrobial activity. Molecular identification showed that the active fungi could be classified as Sordariomycetes and identified as *N. guilinensis*; which was also reported to be the major endophytic group from various plant species (Zhang and Yao, 2015). *Nigrospora* spp. was commonly found as an endophyte in several species of plants (Sharma and Rangari, 2015; Tenguria and Firodiya, 2015; Kucerova-Chlupacova et al., 2016; Saad et al., 2019). Furthermore, there are many reports about metabolites from *Nigrospora* spp. (Arumugam et al., 2014; Rathod et al., 2014; Ibrahim et al., 2018) that displayed good activity against pathogenic micro-organisms. Some secondary metabolites (griseofulvin, spirobenzofuran and pyrazine) from *Nigrospora* spp. have been reported as antifungal and antibacterial substances (Kratky et al., 2012; Roymahapatra et al., 2012; Sharma and Rangari, 2015; Kucerova-Chlupacova et al., 2016). In addition, the ethyl acetate extract of *N. guilinensis* (TSU-EFHA009) contained three main compounds which are 4-(cyclopentyloxy)cyclohex-2-en-1-yl acetate, 5,7a-dimethyloctahydro-1-inden-3a-yl)(phenyl) methanone and 2-methylcyclohexanone. It is possible that they might have an important role in antimicrobial and antioxidant activities. However, these compounds have not been previously reported from *Nigrospora* spp. and there are no reports about their biological activities.

Phenolic compounds are secondary metabolites that stabilize lipid oxidation. The amount of phenolic content in crude extracts seems to have an important role in antioxidant activity. The total phenolic content of fungal extracts has been previously determined (Bharwaj et al., 2015; Madhuchhanda et al., 2017). Total phenolic content in this study ranged from 4.30±0.25 to 41.20±0.40 gallic acid equivalents (GAE mg/g of extract) of dry weight of extracts (Table 2). Total phenolic content was lower than the extracts of endophytic fungi from *Eugenia jambolana.* The endophytic extracts having high phenolic contents showed a high antioxidant activity which ranged from 58 to 60 GAE mg/g of extract and produced a 50 to 80% inhibition (Yadav et al., 2014). The antioxidant content range in this study was different from previous studies

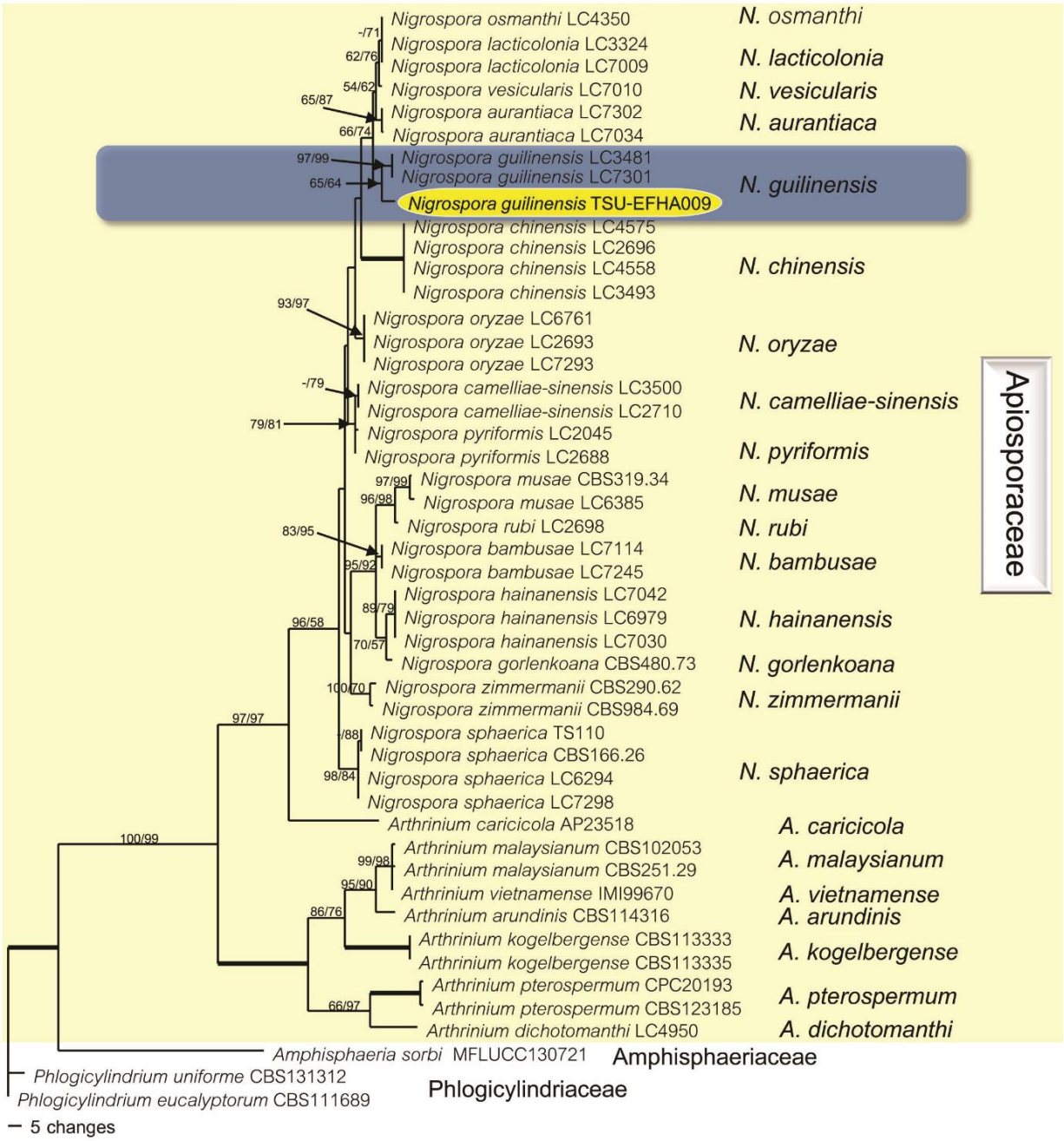

Figure 2. The most parsimonious trees using the ITS rDNA gene with *Amphisphaeria sorbi* (MFLUCC 13-0721), *Phlogicylindrium eucalyptorum* (CBS111689) and *P. uniforme* (CBS131312) as outgroup. The best phylogeny performed by maximum parsimony analyses. Maximum parsimony bootstrap values (BSMP, left) and maximum likelihood (BSML, right) bootstrap values equal or greater than 50% are shown above each branch. The bold line shows strong supported by all bootstrap values (100%).

Table 3. Major constituents of broth ethyl acetate (BE) fraction of *N. guilinensis*.

No.	Compound	% Peak area
1	4-(cyclopentyloxy)cyclohex-2-en-1-yl acetate	21.89
2	5,7a-dimethyloctahydro-1-inden-3a-yl)(phenyl)methanone	12.37
3	2-methylcyclohexanone	10.02

Table 4. GenBank accession number of generated ITS rDNA sequences.

Species	Voucher/Culture	GenBank accession numbers
		ITS rDNA
Arthrinium arundinis	CBS114316	KF144884
Arthrinium caricicola	AP23518	MK014871
Arthrinium dichotomanthi	LC4950	KY494697
Arthrinium kogelbergense	CBS113333	KF144892
Arthrinium kogelbergense	CBS113335	KF144893
Arthrinium malaysianum	CBS102053	KF144896
Arthrinium malaysianum	CBS251.29	KF144897
Arthrinium pterospermum	CPC20193	KF144913
Arthrinium pterospermum	CBS123185	KF144912
Arthrinium vietnamense	IMI99670	KX986096
Nigrospora aurantiaca	LC7034	KX986093
Nigrospora aurantiaca	LC7302	KX986064
Nigrospora bambusae	LC7114	KY385307
Nigrospora bambusae	LC7245	KY385305
Nigrospora camelliae-sinensis	LC2710	KX985957
Nigrospora camelliae-sinensis	LC3500	KX985986
Nigrospora chinensis	LC3493	KX985984
Nigrospora chinensis	LC4558	KX986020
Nigrospora chinensis	LC4575	KX986023
Nigrospora chinensis	LC2696	KX985947
Nigrospora gorlenkoana	CBS480.73	KX986048
Nigrospora guilinensis	LC3481	KX985983
Nigrospora guilinensis	LC7301	KX986063
Nigrospora guilinensis	TSU- EFHA 009	MK033475
Nigrospora hainanensis	LC6979	KX986079
Nigrospora hainanensis	LC7042	KX986094
Nigrospora hainanensis	LC7030	KX986091
Nigrospora lacticolonia	LC3324	KX985978
Nigrospora lacticolonia	LC7009	KX986087
Nigrospora musae	CBS319.34	KX986076
Nigrospora musae	LC6385	KX986042
Nigrospora osmanthi	LC4350	KX986010
Nigrospora oryzae	LC2693	KX985944
Nigrospora oryzae	LC7293	KX985931
Nigrospora oryzae	LC6761	KX986056
Nigrosora pyriformis	LC2688	KX985941
Nigrospora pyriformis	LC2045	KX985940
Nigrospora rubi	LC2698	KX985948
Nigrospora sphaerica	CBS166.26	MH854878
Nigrospora sphaerica	LC6294	KX986044
Nigrospora sphaerica	LC7298	KX985937
Nigrospora sphaerica	TS-110	MG832530
Nigrospora vesicularis	LC7010	KX986088
Nigrospora zimmermanii	CBS290.62	KY385309
Nigrospora zimmermanii	CBS984.69	KY385310
Outgroup		
Amphisphaeria sorbi	MFLUCC 13-0721	KR092797
Phlogicylindrium eucalyptorum	CBS111689	KF251205
Phlogicylindrium uniforme	CBS131312	JQ044426

Bold letters: Strain in this study; AP: culture collection of Rene Jarling, Germany; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IMI: Culture Collection of CABI Europe UK Centre, Egham, UK; KFRD: culture from kiwifruit, Chenzhou, China; LC: culture collection of Lei Cai, China; MFLUCC: Mae Fah Luang University Culture Collection, Chiangrai, Thailand; TS: culture from kiwifruit, Taishun, China.

and this may be due to the fungal strains and extraction method (Srinivansan et al., 2010; Chowdhury et al., 2018). However, the results in this study confirmed that the endophytic fungus (TSU-EFHA009) has a high phenolic content and showed excellent activity against DPPH radicals.

Conclusions

Broth ethyl acetate extract of endophytic fungus *N. guilinensis* TSU-EFHA009 significantly showed strong antimicrobial and antioxidant activities. This finding confirms that endophytic fungi isolated from *C. carandas* were sources of the potential substances. Thus, this plant appears to be an interesting plant which harbors active fungal isolates for development as pharmaceutical agents in the future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Arif M, Kamal M, Jawaid T, Khalid, M, Saini KS, Kumar A, Ahmad M (2016). *Carissa carandas* Linn. (Karonda): an exotic minor plant fruit with immense value in nutraceutical and pharmaceutical industries. Asian Journal of Biomedical and Pharmaceutical Science 6:14-19.

Arumugam GK, Srinivasan SK, Joshi G, Gopal D, Ramalingam K (2014). Production and characterization of bioactive metabolites from piezo tolerant deep sea fungus *Nigrospora* sp. in submerged fermentation. Journal of Applied Microbiology 118:99-111.

Ascencio PGM, Ascencio SD, Aguiar AA, Fiorini A, Pimenta RS (2014). Chemical assessment and antimicrobial and antioxidant activities of endophytic fungi extracts isolated from *Costus spiralis* (Jacq.) Roscoe (Costaceae). Evidence Based Complementary and Alternative Medicine 2014:190543.

Beveridge TJ (1999). Structure of Gram-negative cell walls and their derived membrane vesicles. Journal of Bacteriology 181:4725-4733.

Bharwaj A, Sharma D, Jadon N, Agrawal PK (2015). Antimicrobial and phytochemical screening of endophytic fungi isolated from spikes of *Pinus roxburghii*. Archives of Clinical Microbiology 6:1-9.

Brissow ER, da Silva IP, de Sigueira JA, Pimenta LP, Januario AH, Magalhaes LG, Furtado RA, Tavares DC, Junior PA, Santos JL, Soares MA (2017). 18-Des-hydroxy cytochasin: an antiparasitic compound of *Diaporthe phaseolorum*-92c, an endophytic fungus isolated from *Combretum lanceolatum* Pohl ex Eichler. Parasitology Research 116:1823-1830.

Chowdhury DR, Chattopadhyay SK, Roy SK (2018). Assessment of secondary metabolites with relation to their antioxidant activity of fungal endophytes isolated from medicinal plants. International Journal of Pharmaceutical Sciences 10:59-63.

Clinical and Laboratory Standards Institute (CLSI) (2008). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi. 2nd Edn. Clinical and Laboratory Standards Institute, Wayne, PA., USA.

CLSI (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. 9th Edn. Clinical and Laboratory Standards Institute, Wayne, PA., USA.

Edgar RC (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32:1792-1797.

Gouda S, Das G, Sen SK, Shin HS, Patra JK (2016). Endophytes: A treasure house of bioactive compounds of medical importance. Frontiers Microbiology 7:1538.

Hall T (2005) BioEdit: Biological Sequence Alignment Editor for Windows 95/98/NT/XP. Available: http://www.mbio.ncsu.edu/bioedit/page1.html.

Huang WY, Cat YZ, Xing J, Corke H, Sun M (2007). A potential antioxidant resource: endophytic fungi from medicinal plants. Economic Botany 61:14-30.

Hubalek Z (2003). Emerging human infectious disease: anthroponoses, Zoonoses, and sapronoses. Emerging Infection Disease 9:403-404.

Ibrahim D, Lee CC, Yenn TW, Zakaria L, Sheh-Hong L (2015). Effect of the extract of endophytic fungus, *Nigrospora sphaerica* CL-OP 30, against the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumonia* cells. Tropical Journal of Pharmaceutical Research 14:2091-2097.

Ibrahim SRM, Abdallah HM, Elkhayat ES, Musayeib NM, Zayed MF, Mohamed GA (2018). Fusaripeptide A: new antifungal and anti-malarial cyclodepsipeptide from the endophytic fungus *Fusarium* sp. Journal of Asian Natural Products Research 20:75-85.

Jalgaonwala RE, Mohite BV, Mahajan RT (2011). A review: natural products from plant associated endophytic fungi. Journal of Microbiology and Biotechnology Research 1:21-32.

Jia M, Chen L, Xin HL, Zheng CJ, Rahman K, Han T, Qin LP (2016). A friendly relationship between endophytic fungi and medical plants: a systematic review. Frontiers Microbiology 7:906.

Joseph B, Priya RM (2011). Bioactive compounds from endophytes and their potential in pharmaceutical effect: a review. American Journal of Biochemistry and Molecular Biology 1:291-309.

Khiralla A, Mohamed I, Thomas J, Mignard B, Spina R, Yagi S, Laurain-Mattar D (2015). A pilot study of antioxidant potential of endophytic fungi from some Sudanese medicinal plants. Asain Pacific Journal of Tropical Medicine 8:701-704.

Kishino H, Hasegawa M (1989). Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in hominoidea. Journal of Molecular Evolution 29:170-179.

Kratky M, Vinsova J, Buchta V (2012). *In vitro* antibacterial and antifungal of salicylanilide pyrazibe-2-carboxylates. Medical Chemistry 8:732-741.

Kucerova-Chlupacova M, Vyskovska-Tyllova V, Richterova-Finkova L, Kunes J, Buchta V, Vejsova M, Paterova P, Semelkova L, Jandourek O, Opletalova V (2016). Novel halogenated pyrazine-based chalcones as potential antimicrobial drugs. Molecules 21:E1421.

Leylaie S, Zafari D (2018). Antiproliferative and antimicrobial activities of secondary metabolites and phylogenetic study of endophytic *Trichoderma* species from *Vinca* plants. Frontiers Microbiology 9:1484.

Lindahl J, Grace D (2015). The consequences of human actions on risks for infectious diseases: a review. Infection Ecology and Epidemiology 5:30048.

Lobo V, Patil A, Phatak A, Chandra N (2010). Free radicals, antioxidants and functional foods: impact on human health. Pharmacognosy Reviews 4:118-126.

Madhuchhanda D, Harischandra P, Somaiah NM (2017). Antioxidant properties of phenolic compounds isolated from the fungal endophytes of *Zingiber nimmonii* (J. Graham) Dalzell. Frontiers in Biology 12:151-162.

Miller MA, Pfeiffer W, Schwartz T (2010). Creating the CIPRES science gateway for inference of large phylogenetic trees. Gateway Computing Environments Workshop (GCE), IEEE, San Diego, Supercomputer Center, La Jolla, CA, USA 14:1-8.

Nisa H, Kamili AN, Nawchoo IA, Shafi S, Shameem N, Bandh SA (2015). Fungal endophytes as profile source of phytochemicals and other bioactive natural products: a review. Microbial Pathogenesis 82:50-59.

O'Donnell K, Cigelnik E, Weber NS, Trappe JM (1997). Phylogenetic relationships among Ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. Mycologia 89:48-65.

Pawle G, Singh SK (2014). Antimicrobial, antioxidant activity and phytochemical analysis of an endophytic species of *Nigrospora* isolated from living fossil *Ginkgo biloba*. Current Research in Environmental and Applied Mycology 4:1-9.

Racek J, Holecek V, Sedlacek D, Panzner P (2001). Free radicals in

immunology and infectious diseases. Epidemiology Microbiology Immunology 50:87-91.
Rambaut A (2016). FigTree (Tree Figure Drawing Tool) Version1.4.3 2006–2016. Institute of Evolutionary Biology, University of Edinburgh. Available: http://tree.bio.ed.ac.uk/software/figtree/
Rathod DP, Dar M, Gade AK, Rai, MK (2014). Griseofulvin producing endophytic *Nigrospora oryzae* from Indian *Emblica officinalis* Gaertn: a new report. Austin Journal of Biotechnology and Bioengineering 1:1-5.
Raunsai M, Wulansari D, Fathoni A, Agusta A (2018). Antibacterial and antioxidant activities of endophytic fungi extracts of medicinal plants from Central Sulawesi. Journal of Applied Pharmaceutical Science 8:069-074.
Roymahapatra G, Mandal SM, Porto WF, Samanta T, Giri S, Dinda J, Franco OL, Chattaraj PK (2012). Pyrazine funtionalized Ag9I) and Au (I)-NHC complexes are potential antibacterial agents. Current Medicinal Chemistry 19:4184-4193.
Saad MMG, Ghareeb RY, Saeed AA (2019). The potential of endophytic fungi as bio-control agents against the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). Egyptian Journal of Biological Pest Control 29:1-7.
Sharma A, Rangari V (2015). Antibacterial, antifungal and antitubercular activity of methanolic extracts of *Adansonia digitate* L. Journal of Pharmacy and Biological Sciences 10:52-60.
Srinivansan K, Jagadish LK, Shenbhagaraman R, Muthumary J (2010). Antioxidant activity of endophytic fungus *Phyllosticta* sp. isolated from *Guazuma tomentosa*. Journal of Phytology 2:37-41.
Stamatakis A (2014). RAxML version 8: a tool for phylogenetic analysis and postanalysis of large phylogenies. Bioinformatics 30:1312-1313.
Supaphon P, Kaewpiboon C, Preedanon S, Phongpaichit S, Rukachaisirikul V (2018). Isolation and antimicrobial activities of fungi derived from *Nymphaea lotus* and *Nymphaea stellata*. Mycoscience 59:415-423.
Supaphon P, Phongpaichit S, Rukachaisirikul V, Sakayaroj J (2013). Antimicrobial potential of endophytic fungi derived from three seagrass species: *Cymodocae serrulata, Halophila ovalis* and *Thalassia hermprichii*. Plos One 8:e72520.
Supaphon P, Phongpaichit S, Rukachaisirikul V, Sakayaroj J (2010). Diversity and antimicrobial activity of endophytic fungi isolated from seagrass *Enhalus acoroides*. Indian Journal *of* Marine *Sciences* 43:785-797.
Swofford DL (2002). PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.
Tenguria RK, Firodiya A (2015). Occurrence of endophytic fungi in leaves of medicinal plants from central region of Madhya Pradesh, India. World Journal of Pharmacy and Pharmaceutical Sciences 4:1921-1934.
Tenguria RK, Firodiya A (2016). Cytotoxic activity of endophytic fungi isolated from central region of Madhya Pradesh. Journal of Innovations in Pharmaceuticals and Biological Sciences 3:29-38.
Tenguria RK, Firodiya A, Khan FN (2012). Biodiversity of endophytic fungi in leaves of *Carissa carandas* Linn. From central region of Madhya Predesh. Pharmaceutical Technology 3:376-380.
Toobpeng N, Powthong P, Suntronthiticharoen P (2017). Evaluation of antioxidant and antibacterial activities of fresh and freeze-dried selected fruit juices. Asian Journal of Pharmaceutical and Clinical Research 10:156-160.
Wang M, Liu F, Crous PW, Cai L (2017). Phylogenetic reassessment of *Nigrospora*: ubiquitous endophytes, plant and human pathogens. Persoonia 39:118-142.
White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA gene for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JS, White TJ (eds), PCR Protocol: A guide to methods and applications. Academic, San Diego pp. 315-322.
Yadav M, Yadav A, Yadav JP (2014). *In vitro* antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana*. Asian Pacific Journal of Tropical Medicine 7:256-261.
Zhang T, Yao Y (2015). Endophytic fungi communities associated with vascular plants in the high arctic zone are highly diverse and host-plant specific. Plos One 10:e0130051.