

Analysis of chemical constituents, antimicrobial and anticancer activities of dichloromethane extracts of *Sordariomycetes* sp. endophytic fungi isolated from *Strobilanthes crispus*

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Abstract Plants are primary source of natural product drugs. However, with every new bioactive molecule reported from a plant source, there follows reports of endangered status or even extinction of a medicinally important plant due to over-harvesting. Hence, the attention turned towards fungi namely the endophytes, which reside within medicinally important plants and thus may have acquired their medicinal properties. *Strobilanthes crispus* is a traditional medicinal plant which has been used traditionally to treat kidney stones, diabetes, hypertension and cancer as well as having antimicrobial activities. In our efforts to bioprospect for anticancer and antimicrobial metabolites, two fungal endophytes most closely related to the *Sordariomycetes* sp. showed promising results. Sample (PDA)BL3 showed highest significant antimicrobial

activity against 6 bacteria at 200 µg/disc whereas sample (PDA)BL5 has highest significant anticancer activity against all 5 cancer cell lines at concentrations ranging from 30 to 300 µg/ml. As for the gas chromatography coupled with mass spectrometry (GC–MS) results, a total of 20 volatile metabolites identified from sample (PDA)BL3 and 21 volatile metabolites identified from sample (PDA)BL5 having more than 1% abundance. Both GC–MS analysis showed that compound Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) has the highest abundance at 15.10% abundance for sample (PDA)BL3 and 19.00% abundance for sample (PDA)BL5 respectively. In conclusion, these results have shown bioprospecting potential of endophytic fungi having antimicrobial and anticancer activities as well as its potential secondary metabolites of interest. Therefore, this work has further indicated the medicinal and industrial potential of endophytic fungi.

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Introduction

Today the range of drugs derived from fungi stretches from antibiotics to supplements to anticancer drugs. Plants still remain the main source of drugs or their lead molecules. However, with every new bioactive molecule reported from a plant source, there follows reports of endangered status or even extinction of a medicinally important plant which is due to over-harvesting. Hence, the attention turned towards fungi namely the endophytes, which reside within medicinally important plants and thus may have

acquired their medicinal abilities (Venugopalan and Srivastava 2015). The landmark in this area of endophyte bioprospecting began from the discovery of *Taxomyces andreanae*, an endophytic fungus of *Taxus brevifolia* (Pacific Yew tree) which was found to produce taxol. Taxol also known as paclitaxel is the world's multi-billion dollar anticancer drug used up to date (Strobel and Daisy 2003).

As an area with over 15,000 plant species, Malaysia could serve as an important source for the host interaction study of endophytic fungi and bacteria screening of tropical rain forest plant and medicinal plants (Radu and Kqueen 2002). The plant of interest would be the *Strobilanthes crispus* which is known traditionally to have medicinal properties to treat kidney stones, diabetes, hypertension, management of cancer as well as to enhance the immune system (Hussin 2006; Samuel et al. 2010). Apart from that, previous studies have also shown that extracts of *Strobilanthes crispus* showed positive results for antimicrobial activities (Koay et al. 2013). Previous studies based on *Strobilanthes crispus* have greatly reviewed its biological potential activity, but analysis based on the fungal endophytes that coexist within the plant has yet been done. Recent reports estimate the presence of about 1.5 million fungal species worldwide. Among these, around 1% species have been analyzed for their spectrum of secondary metabolites (Weber et al. 2007). The term 'secondary metabolism' denotes the biochemical processes underlying the formation of fungal metabolites, such as toxins, antibiotics, and immune-suppressing agents (Strobel et al. 2004; Hoffmeister and Keller 2007).

Ribosomal RNA (rRNA) genes are the most ubiquitous and conserved DNA sequences found in nature (Raue et al. 1988). As a piece of non-functional RNA, 18S rRNA is situated at the same precursor transcript with the structural rRNA. Gene encoded the rRNA normally occurs in tandem repeats and high copy numbers. For this reason, they have been extensively used for phylogenetic analysis (Wagele and Rodding 1998). In our research, two endophytic fungi *Sordariomycetes* sp. showed promising antimicrobial and anticancer activities respectively. The class *Sordariomycetes* is one of the largest monophyletic clades in the *Ascomycota* with more than 1000 genera and 3000 known species (Ainsworth 2008; Maharachchikumbura et al. 2015).

One class of molecules produced by endophytes that are of particular interest is volatile organic compounds (VOCs) (Korpi et al. 2009). These are typically low molecular weight compounds including alcohols, ketones, esters, acids, and hydrocarbons that can be derived from either biosynthetic or degradative pathways (Shaw et al. 2015). The determination of volatile compounds is usually carried out by gas chromatography–mass spectrometry (GC–MS) because of their powerful separation capability and highly

sensitive detection performance. Volatile compounds are known to belong to numerous structure classes, such as hydrocarbons, esters, ethers, alcohols, ketones, lactones, and glycol ethers (Korpi et al. 2009). Less volatile compounds and reactive compounds such as amines, phenols, aldehydes, and unsaturated hydrocarbons are not recovered efficiently due to their strong adsorption to the column material.

In this study, we aim to isolate endophytic fungi samples having antimicrobial and anticancer activities as well as detect a wide range of volatile compounds from the isolated endophytic fungi cultures having activities by the application of gas chromatography–mass spectrometry analysis.

Materials and methods

Plant material

Strobilanthes crispus plant samples were randomly collected on April 2013 from TKC Herbal Nursery, Negeri Sembilan. The botanical identification of *Strobilanthes crispus* was characterized by the Phytomedicinal Herbarium, Institute of Bioscience, Universiti Putra Malaysia, Selangor and were deposited with the voucher number (SK2281/13).

Culturing and isolation of fungal endophytes from plant sample

Endophytes are microorganisms that live within the plant. Thus, each part of the plant collected was subjected to a standard surface-sterilized to eliminate epiphytic microorganisms. Samples was thoroughly washed under running tap water, after which the surface was sterilized by submerging them in 75% ethanol for 2 min, 5.3% sodium hypochlorite for 5 min, and 75% ethanol for 0.5 min, and finally rinsed with sterile distilled water for 1 min. Next, the samples were dried on a sterile filter paper. Each plant tissues were then cut with a sterile blade into 1-cm segments. The segments were placed on potato dextrose agar (PDA), exposing their inner tissue surface. To inhibit bacterial growth, media enriched with antibiotic of 50 mg/l of ampicillin were used. Petri dishes were kept in an aseptic condition and incubated at room temperature (RT) for up to 3 weeks or until the emergence of fungal colonies was observed. Emerging fungi were then transferred to fresh medium by the hyphae tips method to obtain the single isolate. Each isolate was kept in a storage tube for future applications. Culturing and isolation methods were done using a slight modification of the protocol (Sim et al. 2010).

Genomic DNA extraction

Each isolated fungal endophyte was cultured in malt extract broth (MEB) (Difco, USA) and was kept in a shaking incubator at 27 °C. Solid fungi cell tissues obtained were then subjected to a freeze-drying process before extracting genomic DNA using the sodium dodecyl sulfate (SDS) method. The freeze dried solid fungi cell tissues were grinded using a mortar and pestle to attain powdery form. The fungi powdery forms were collected into microcentrifuge tubes. Extraction buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5 M NaCl, and 1% SDS) with 0.2 g/ml of starting tissue was added into each microcentrifuge tube, vortexed for 10 s and incubated at room temperature for 30 min. Subsequently, 0.4 g/ml of phenol: chloroform: isoamylalcohol (25:24:1) was added to the mixture and centrifuged at 13,000 rpm for 10 min at 4 °C. The first layer from the top was transferred to a new microcentrifuge tube where equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added to the mixture and centrifuged at 13,000 rpm for 10 min at 4 °C. Once again the first layer from the top was then transferred to a new microcentrifuge tube, where 2–3 µL RNase A (20 mg/mL) was added to the mixture and incubated at 37 °C for 15 min. Next, the mixture was mixed with 1/2 volume 7.5 M ammonium acetate and 1 volume of isopropanol (ice cold) with gently inversion and incubated at –20 °C for 30 min. The mixture was centrifuged at 13,000 rpm for 15 min at 4 °C. The resulted pellet of DNA was rinsed with 70% ethanol. The mixture was centrifuged at 13,000 rpm for 15 min at 4 °C. The 70% ethanol was gently poured out and the resulting pellet was air dried. The dried pellet was re-suspended in 100 µl of ultrapure water. The extracted DNA were then stored at –20 °C. DNA yield and purity were measured using the NanoPhotometer spectroscopy system (Implen, Munchen, Germany). Genomic DNA extraction method was done using a slight modification of the protocol (Sim et al. 2010).

Amplification of 18S rRNA sequences

The partial nucleotide base-pair fragment of the 18S rDNA gene from the isolated fungal endophytes were amplified using the polymerase chain reaction (PCR) with universal 18S primers NS1 (5' GTA-GTC-ATA-TGC-TTG-TCT-C 3') and NS8 (5' TCC-GCA-GGT-TCA-CCT-ACG-GA 3') (Berbee and Taylor 1992; Smithee et al. 2014). The PCR amplification reactions were performed in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with a total 25 µl of reaction that comprised 20 ng of genomic DNA, 2.5 µl of 10× PCR buffer with 25 mM MgCl₂, 2.0 µl of 10 mM dNTPs, 1.25 µl of 5% (v/v) DMSO, 1 unit of Taq polymerase (GeNet Bio, Korea), and 10 pmol of each

primer. A non-template control was included in each run. A touchdown PCR amplification programme was used whereby cycling parameter were 5 min at 95 °C for pre-denaturation, then touchdown PCR for 9 cycles each of 1 min at 95 °C for denaturation, 50 s at decreasing 1 °C for each cycle from 54 to 46 °C for annealing and 1.5 min at 72 °C for extension, this is followed by 31 cycles each of 1 min at 95 °C for denaturation, 50 s at 45 °C for annealing and 1.5 min at 72 °C for extension, with a final extension of 5 min at 72 °C (Gahan and Schmalenberger 2015). The amplified DNA was sequenced commercially (First-base, Seri Kembangan, Malaysia). The sequence obtained from each isolate was further analyzed using BLAST [National Center for Biotechnology Information (NCBI)]. The sequences of the 18S rRNA regions were aligned with those of related fungal strains retrieved from the GenBank databases using ClustalX. A phylogenetic tree was constructed from the evolutionary distance data using MEGA software version 6.06 (Kumar et al. 2008). Sequences obtained and reported in the present study were deposited in the GenBank database of NCBI with the accession numbers KY020350 for sample (PDA)BL3 and KY020351 for sample (PDA)BL5 respectively.

Culturing and semipolar extraction of secondary metabolites from fungal endophytes

Many discoveries have been made in isolating fungal endophytes, which have been shown to have the potential for de novo synthesis of various bioactive metabolites that may directly or indirectly be used as therapeutic agents against numerous ailments (Strobel and Daisy 2003; Kharwar et al. 2011). Secondary metabolites for fungal endophytes isolated were extracted using the dichloromethane solvent. Fungal endophytes were cultured in malt extract broth (MEB) (Difco, USA) of 400 ml in a 1000 ml conical flask for 21 days. The cultures underwent continuous shaking to allow optimum growth of endophytes. The broth cultures were then filtered through filter papers (Whatman, Maidstone, UK) to remove the unwanted hyphae. The broth mixture collected was then extracted using Dichloromethane solvents (Merck, Kenilworth, NJ, USA) in the ratio of 1:1. The mixtures were shook vigorously to ensure optimum extraction of secondary metabolites from the aqueous phase in the broth. This is followed by separation of solvent and broth extracts using the separating funnel. However, assurance of complete solvent and broth extracts separation was needed by letting the mixture settle and set completely to two difference phase before separation. The solvents extracted from different fungal endophyte isolates were then transferred to a round bottom flask and dried using a rotary evaporator (Cole Parmer, Illinois, USA). The resultant extracts were

dissolved in 1 ml of dimethyl-sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, Missouri, USA).

Antimicrobial activity

Antimicrobial activities were measured using a disc diffusion method on selected pathogenic and nonpathogenic bacteria from the American type culture collection (ATCC). The secondary metabolite crude extracts were prepared as mentioned previously and were used for the test. The selected bacteria are divided into gram-positive and gram-negative bacteria. 8 g-positive bacteria were used which are *Bacillus cereus* (ATCC 10876), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Enterococcus faecium* (ATCC 19434), *Listeria monocytogenes* (ATCC 19117), *Staphylococcus aureus* (ATCC 25923), Methicillin-resistant *Staphylococcus aureus* (ATCC 43300) and *Staphylococcus saprophyticus* (ATCC 15305). Whereas, 16 g-negative bacteria were used which are *Acinetobacter baumannii* (ATCC 19606), *Aeromonas hydrophila* (ATCC 35654), *Citrobacter freundii* (ATCC 8090), *Enterobacter aerogenes* (ATCC 13048), *Enterobacter cloacae* (ATCC 35030), *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 700603), *Proteus mirabilis* (ATCC 25933), *Proteus vulgaris* (ATCC 13315), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella paratyphi A* (ATCC 9150), *Salmonella typhimurium* (ATCC 14028), *Shigella flexneri* (ATCC 12022), *Shigella sonnei* (ATCC 9290), *Stenotrophomonas maltophilia* (ATCC 13637) and *Vibrio parahaemolyticus* (ATCC 17802). The bacteria were cultured overnight at 30–37 °C in tryptic soy broth (TSB) (Difco, USA) to an optical density (OD) of 0.7 at 600 nm. It is then diluted to an optical density (OD) 0.1 at 600 nm. 100 ml of diluted concentration bacteria culture of was spread evenly on mueller–hinton agar (MHA) (Difco, USA) plates for further assessment. Blank sterile discs (Oxoid, Basingstoke, UK) were placed on the MHA petri dishes and 10 µl of the fungal endophyte secondary metabolite crude extracts were then added onto the blank disc. The assays were conducted in triplicates and repeated two times. The plates were incubated at 30–37 °C accordingly for 24 h. Antimicrobial activities were determined by measuring the inhibition zone of the fungal endophyte's crude extract against selected pathogens. Tetracycline (Oxoid, Basingstoke, UK) which is an established antibiotic against bacteria was used for comparison. The values of antimicrobial activities were expressed as mean ± SD for three replicate. One-way ANOVA and Post-hoc testing was performed for inter grouping comparisons using the Tukey's Test (SPSS), where values of $p < 0.001$ are significant.

Anticancer and cytotoxic activity

Anticancer and cytotoxic activity of the fungal endophyte's secondary metabolite crude extracts were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, Missouri, USA) and CellTiter96 Aqueous One Solution Cell Proliferation assay (Promega, Madison, Wisconsin, USA) on 5 human cancer cell line and 1 normal cell line obtained from the ATCC. The secondary metabolite crude extracts were prepared as mentioned previously. The cell lines are human prostatic adenocarcinoma cells, PC-3 (ATCC CRL-1435), human hepatocellular carcinoma cell, HEPG2 (ATCC HB-8065), human alveolar adenocarcinoma cells, A549 (ATCC CCL-185), human colorectal adenocarcinoma cells, HT-29 (ATCC HTB-38), human breast adenocarcinoma cells, MCF-7 (ATCC HTB-22) and human embryonic kidney 293 cells, HEK-293 (ATCC CRL-1573). All cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO₂. MTT Assay plates were read using Asys UVM 340 Microplate Reader (Biochrom, Cambridge, UK) at 570 nm and reference wavelength at 630 nm. As for CellTiter96 Aqueous One Solution Cell Proliferation Assay, plates were read using Asys UVM 340 Microplate Reader at 490 nm.

The percentage of cell viability (CV) was calculated manually using:

$$CV = \frac{\text{Absorbance of drug wells}}{\text{Absorbance of control wells}} \times 100\%$$

Data generated were used to plot a dose–response curve of which the concentration of extract required to kill 50% of cell population (IC₅₀) was determined. The assays were conducted in triplicates and repeated two times. Doxorubicin (Sigma-Aldrich, St. Louis, Missouri, USA) which are both established chemotherapeutics were used for comparison. The values of anticancer activities were expressed as mean ± SD for three replicate. One-way ANOVA and Post-hoc testing was performed for inter grouping comparisons using the Tukey's Test (SPSS), where values of $p < 0.001$ are significant. Anticancer tests were done using a slight modification of the protocol (Hazalin et al. 2009).

Cell cycle and Annexin-V analysis

Human prostatic adenocarcinoma cells, PC-3 (ATCC CRL-1435), human hepatocellular carcinoma cells were used in this analysis due to significant findings in anticancer activity or cytotoxic activity. PC-3 cells were seeded in 25 cm² cell culture flasks at 5×10^5 cells per ml of complete growth culture media, followed by incubation at 37 °C in an atmosphere of 5% CO₂. After incubation, cells of interest were treated with doxorubicin (IC₅₀ = 20 µg/

ml) and (PDA)BL5 ($IC_{50} = 25 \mu\text{g/ml}$). Untreated control cells with 0.1% DMSO alone were also included. Following incubation, the floating cells were collected. Adherent cells were trypsinised to detach them from the substratum. 1×10^6 cells per ml were used to process cell cycle test using MuseTM Cell Cycle Kit (Merck Millipore, Darmstadt, Germany) and 3×10^5 cells per ml were used to process Annexin-V analysis using MuseTM Annexin-V and Dead Cell Kit (Merck Millipore, Darmstadt, Germany). Results were analysed and obtained using Muse[®] Cell Analyzer (Merck Millipore, Darmstadt, Germany). Cell cycle and Annexin-V analyses were done using modification of the protocol (Hsieh et al. 2014).

Gas chromatography–mass spectrometry (GC–MS) analysis

Separation of hydrocarbons and other volatile compounds were done using a gas chromatograph-mass spectrometry (GC–MS) QP2010 Plus (Shimadzu Lab Solution Japan). GC–MS analysis of dichloromethane extracts of selected endophytic fungi were carried out using medium polar column, BP10 capillary column ($50 \text{ m} \times 0.33 \text{ mm} \times 0.25 \mu\text{m}$). The GC oven temperature was programmed with an initial temperature of $60 \text{ }^\circ\text{C}$ for 2 min. This is followed by a rate of temperature increase of $10 \text{ }^\circ\text{C/min}$ up to $260 \text{ }^\circ\text{C}$. The temperature is held at the final hold at $260 \text{ }^\circ\text{C}$ for 5 min. The injector temperature was maintained at $260 \text{ }^\circ\text{C}$ (splitless) and detector temperature was $280 \text{ }^\circ\text{C}$. GC–MS analyses were conducted with ionization energy of 70 eV. Compounds obtained were putatively identified by mass-spectral database search and matching with the spectra of the MS library. All volatile compounds showing mass spectra with match factors' 90% were included in a 'positive list' of tentatively identified compounds. Gas chromatography–mass spectrometry (GC–MS) analyses were done using a slight modification of the protocol (Siddiquee et al. 2015).

Light microscopic observation

Lactophenol cotton blue mount slides for viewing were prepared before observation under the light microscope. The fungal mount slides were prepared using the scotch tape method. First of all, a drop of lactophenol cotton blue solution (Sigma-Aldrich, St. Louis, Missouri, USA) was placed on a microscopic slide. Next, cut a 2 inch long frosted tape by holding both the ends of the tape and gently tap the top of the fungi isolates on the petri dish. The tape with attached fungal elements from the colony was then dapped on the drop of lactophenol cotton blue solution on the microscopic slide. A coverslip was placed over it. The completed mount slides were viewed and pictures were

taken under observation of the light microscope (Olympus, Tokyo, Japan). Lactophenol cotton blue (LPCB) mount slides were done using a slight modification of the protocol (Harris 2000).

Scanning electron microscope microanalysis

Fungi samples for SEM were processed before observation under the scanning electron microscope (Yamauchi et al. 1990). Fungi samples on agar plates were first of all cut into a number of 1 cm^2 slices and placed into separate vials and were fixed in 4% glutaraldehyde. Next, the samples were washed or rinsed using 0.1 M sodium cacodylate buffer 3 times for 10 min each. Subsequently the samples were post-fixed in 1% osmium tetroxide at $4 \text{ }^\circ\text{C}$ for 2 h. This was followed by the washing process with 0.1 M sodium cacodylate buffer 3 times for 10 min each. Next, the samples underwent a series of dehydration process whereby samples were soaked in 20, 30, 40, 50, 60, 70, 80, 90 and 100% ethanol respectively for 10 min each followed by 100% acetone twice for 15 min. The samples were then transferred into a specimen basket and placed in the critical point dryer Leica EM CPD030 (Leica Microsystem, US) for 45 min. This was followed by the mounting process where specimens were placed onto a stub and coated with gold using a sputter coater Baltec SCD 005 (Baltec, Canonsburg, US). The specimens were then examined with a Jeol JSM-6400 SEM (Jeol, Tokyo, Japan) at 15 kV.

Results

Molecular identification of endophytic fungi by 18S rDNA sequences and phylogenetic analysis

The 18S rRNA is a nonfunctional RNA sequence located in structural ribosomal RNAs (rRNA). Sequence comparison of the 18S region is widely used in taxonomy and molecular phylogeny because of its high copy number of rRNA genes, which allows easy amplification even from small quantities of DNA, and it possesses a high degree of variation even between closely related species. Molecular identification, which was based on sequence analysis, revealed that the isolated endophytic fungi were closely related to 13 genera and 1 class which are *Coniochaeta*, *Lecythophora*, *Phaeoacremonium*, *Togninia*, *Ceratosphaeria*, *Ophiostoma*, *Sporothrix*, *Annulusmagnus*, *Ascitendus*, *Rhodoveronaea*, *Lentomitella*, *Barbatosphaeria* and *Sordariomycetes*. From the phylogenetic tree (Fig. 1), it shows that both the sample (PDA)BL3 and (PDA)BL5 were most closely related to the *Sordariomycetes* sp.

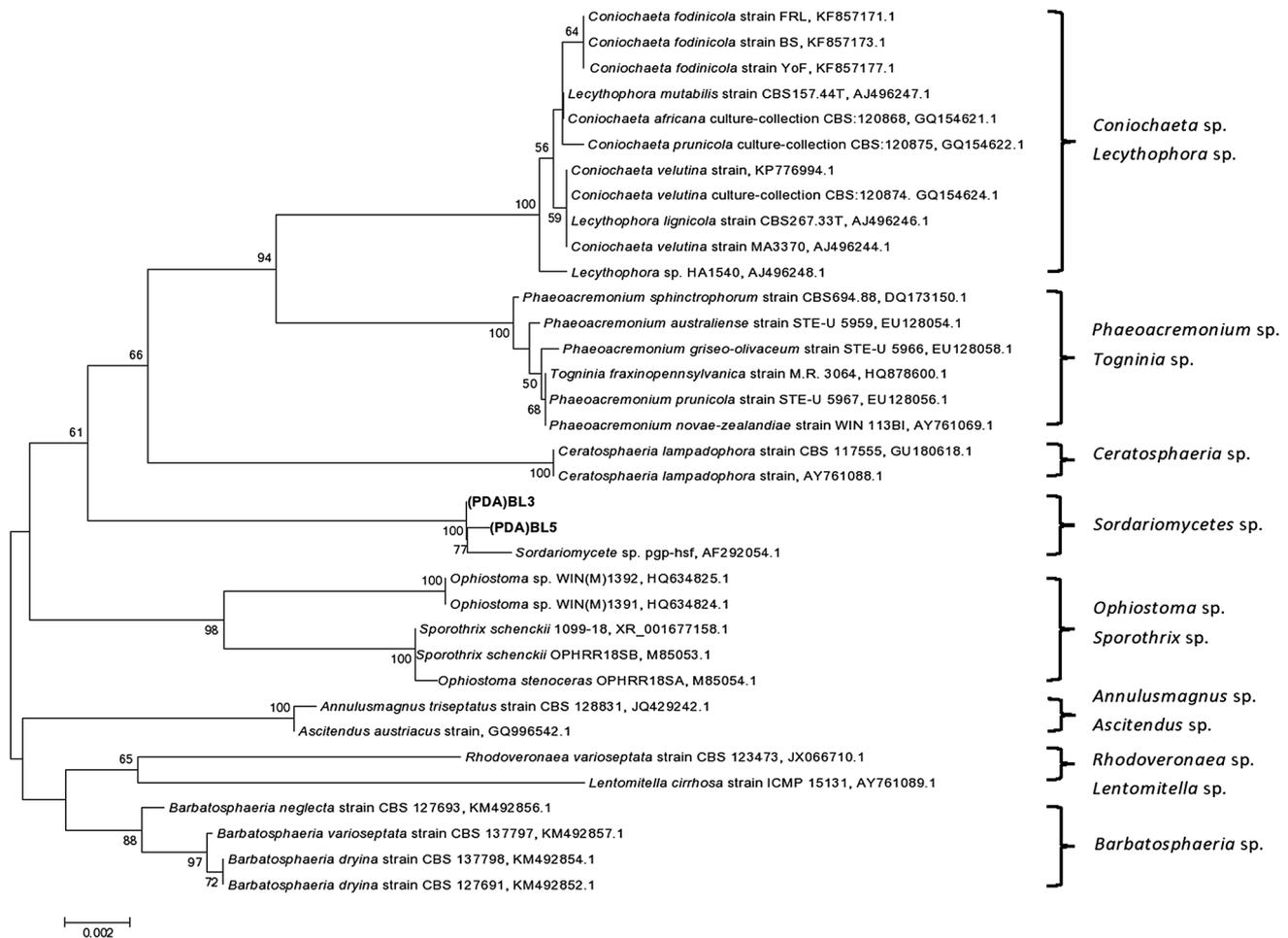


Fig. 1 Neighbor-joining phylogenetic tree based on 18S rRNA gene sequence showing the relationship between (PDA)BL3, (PDA)BL5 strains and representatives or related taxa from Genebank. Numbers

on branches nodes indicate confidence levels from 1000 bootstrap replicates, values above 50% are shown. Scale bar represents 0.002 substitutions per nucleotide position

Antimicrobial activity

The results of antimicrobial activities of disc diffusion inhibition zones (Mean \pm SD) of dichloromethane extract suspensions or secondary metabolites of endophytic fungi sample (PDA)BL3, (PDA)BL5 and their respective controls are shown in Table 1. All screening results of diameter less than 10 mm diffusion zone were eliminated. Results show that endophytic fungi sample (PDA)BL3 expressed antimicrobial activity against 6 g positive bacteria which are *Bacillus cereus* (ATCC 10876), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), Methicillin-resistant *Staphylococcus aureus* (ATCC 43300) and *Staphylococcus saprophyticus* (ATCC 15305). (PDA)BL3 did not show any significant antimicrobial positive results towards the other 2 g-positive bacteria and 16 g-negative bacteria. Whereas, fungal endophyte sample (PDA)BL5 did not express any antimicrobial activity towards all 8 g-

positive bacteria and 16 g-negative bacteria. Apart from that, results show that both the sample (PDA)BL3 and (PDA)BL5 have significant differences with negative and positive controls at $p < 0.001$.

Anticancer and cytotoxic activity

Anticancer and cytotoxic activities against 5 cancer cell lines and 1 normal cell line by dichloromethane extract suspensions or secondary metabolites of endophyte fungi sample (PDA)BL3, (PDA)BL5 and their respective controls are shown in Table 2 for MTT assay tests and Table 3 for CellTiter96 Aqueous One Solution Cell Proliferation assay tests respectively. The IC_{50} value is defined as the concentration of extract required to produce 50% reduction in viability. Any results more than 400 μ g/ml concentration were eliminated and indicated as negative results. Both the assay's results show that endophytic fungi sample (PDA)BL5 exhibited anticancer properties towards all 5

Table 1 Antimicrobial activity of the dichloromethane crude extracts of endophytic fungi (PDA)BL3, (PDA)BL5 and controls

Disc diffusion inhibition zone diameter [(mm) mean \pm SD]				
American type culture collection (ATCC) strains	(PDA)BL3 (200 μ g/disc)	(PDA)BL5 (200 μ g/disc)	Negative control ^a (DMSO)	Positive control ^b Tetracycline (30 μ g/disc)
Gram-positive bacteria				
<i>Bacillus cereus</i> (ATCC 10876)	10.167 \pm 0.289 ^{a,b}	–	–	17.333 \pm 1.041
<i>Bacillus subtilis</i> (ATCC 6633)	10.333 \pm 0.289 ^{a,b}	–	–	30.833 \pm 0.289
<i>Enterococcus faecalis</i> (ATCC 29212)	10.000 \pm 0.500 ^{a,b}	–	–	14.333 \pm 0.577
<i>Enterococcus faecium</i> (ATCC 19434)	–	–	–	23.833 \pm 0.764
<i>Listeria monocytogenes</i> (ATCC 19117)	–	–	–	27.167 \pm 1.041
<i>Staphylococcus aureus</i> (ATCC 25923)	12.833 \pm 0.289 ^{a,b}	–	–	24.167 \pm 0.289
Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) (ATCC 43300)	10.667 \pm 0.289 ^{a,b}	–	–	26.500 \pm 0.500
<i>Staphylococcus saprophyticus</i> (ATCC 15305)	11.333 \pm 0.577 ^{a,b}	–	–	30.667 \pm 0.577
Gram-negative bacteria				
<i>Acinetobacter baumannii</i> (ATCC 19606)	–	–	–	20.167 \pm 0.764
<i>Aeromonas hydrophila</i> (ATCC 35654)	–	–	–	27.167 \pm 0.577
<i>Citrobacter freundii</i> (ATCC 8090)	–	–	–	26.000 \pm 0.500
<i>Enterobacter aerogenes</i> (ATCC 13048)	–	–	–	21.667 \pm 0.577
<i>Enterobacter cloacae</i> (ATCC 35030)	–	–	–	21.667 \pm 0.289
<i>Escherichia coli</i> (ATCC 25922)	–	–	–	23.833 \pm 0.764
<i>Klebsiella pneumoniae</i> (ATCC 700603)	–	–	–	14.667 \pm 0.289
<i>Proteus mirabilis</i> (ATCC 25933)	–	–	–	25.500 \pm 0.500
<i>Proteus vulgaris</i> (ATCC 13315)	–	–	–	27.000 \pm 0.500
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	–	–	–	10.500 \pm 0.500
<i>Salmonella paratyphi A</i> (ATCC 9150)	–	–	–	26.667 \pm 0.289
<i>Salmonella typhimurium</i> (ATCC 14028)	–	–	–	22.000 \pm 0.500
<i>Shigella flexneri</i> (ATCC 12022)	–	–	–	26.000 \pm 0.500
<i>Shigella sonnei</i> (ATCC 9290)	–	–	–	26.333 \pm 0.289
<i>Stenotrophomonas maltophilia</i> (ATCC 13637)	–	–	–	29.000 \pm 0.500
<i>Vibrio parahaemolyticus</i> (ATCC 17802)	–	–	–	23.833 \pm 0.289

^a Values are significantly different from negative control group at $p < 0.001$

^b Values are significantly different from positive control group at $p < 0.001$

cancer cell lines which are human prostatic adenocarcinoma cells, PC-3 (CRL-1435); human hepatocellular carcinoma cell, HEPG2 (HB-8065); human alveolar adenocarcinoma cells, A549 (CCL-185); human colorectal adenocarcinoma cells, HT-29 (HTB-38); and human breast adenocarcinoma cells, MCF-7 (HTB-22). Whereas, fungal endophyte sample (PDA)BL3 showed anticancer properties towards 2 cancer cell lines which are human prostatic adenocarcinoma cells, PC-3 (CRL-1435) and human hepatocellular carcinoma cell, HEPG2 (HB-8065). Apart from that, results show that sample (PDA)BL3 showed significant difference with the positive control, doxorubicin and the negative control (0.1% DMSO) for all cancer cell lines. However, sample (PDA)BL5 showed significant difference

to all negative control and positive control of 4 cancer cell lines. Sample (PDA)BL5 did not show significant difference to positive control for human prostatic adenocarcinoma cells (PC-3).

Cell cycle and Annexin-V analysis

The cell cycle analysis and Annexin-V analysis for human prostatic adenocarcinoma cells (PC-3) are shown in Fig. 2 and 3 respectively. Cell cycle results in Fig. 2 showed that Sub G₀/G₁ phase was detected in cell cycle analysis for PC-3 cells treated with doxorubicin which acts as the positive control at 26.7% and sample (PDA)BL5 at 30.2% respectively. This phase indicate the presence of apoptotic

Table 2 IC₅₀ activity of the dichloromethane crude extracts of endophytic fungi (PDA)BL3, (PDA)BL5 and controls of MTT assay analysis

Cytotoxic activity IC ₅₀ [(μg/mL) mean ± SD]					
American type culture collection (ATCC) cells		(PDA)BL3 ^a	(PDA)BL5 ^b	Positive control ^c Doxorubicin (μg/ml)	Negative control* (0.1% DMSO)
Human prostatic adenocarcinoma cells	PC-3 (CRL-1435)	367.667 ± 2.517 ^{*,b,c}	27.667 ± 1.528 ^{*,a}	19.667 ± 1.528 ^{*,a}	–
Human hepatocellular carcinoma cell	HEPG2 (HB-8065)	358.667 ± 2.517 ^{*,b,c}	148.333 ± 2.517 ^{*,a,c}	8.000 ± 1.000 ^{*,ab}	–
Human alveolar adenocarcinoma cells	A549 (CCL-185)	–	147.667 ± 1.528 ^{*,a,c}	20.667 ± 1.528 ^{*,a,b}	–
Human colorectal adenocarcinoma cells	HT-29 (HTB-38)	–	31.000 ± 1.000 ^{*,a,c}	4.667 ± 0.577 ^{*,a,b}	–
Human breast adenocarcinoma cells	MCF-7 (HTB-22)	–	161.667 ± 1.528 ^{*,a,c}	1.333 ± 0.577 ^{*,a,b}	–
Human embryonic kidney 293 cells	HEK-293 (CRL-1573)	–	–	21.000 ± 1.000 ^{*,a,b}	–

* Values are significantly different from negative control group at $p < 0.001$

^{a,b,c} Values are significantly different when compared between samples of each test groups at $p < 0.001$

Table 3 IC₅₀ activity of the dichloromethane crude extracts of endophytic fungi (PDA)BL3, (PDA)BL5 and controls of CellTiter96 Aqueous One Solution Cell Proliferation assay analysis

Cytotoxic activity IC ₅₀ [(μg/mL) mean ± SD]					
American type culture collection (ATCC) cells		(PDA)BL3 ^a	(PDA)BL5 ^b	Positive control ^c Doxorubicin (μg/ml)	Negative control* (0.1% DMSO)
Human prostatic adenocarcinoma cells	PC-3 (CRL-1435)	355.333 ± 2.517 ^{*,b,c}	23.667 ± 1.155 ^{*,a}	18.667 ± 1.155 ^{*,a}	–
Human hepatocellular carcinoma cell	HEPG2 (HB-8065)	352.667 ± 2.082 ^{*,b,c}	147.000 ± 2.000 ^{*,a,c}	7.667 ± 0.577 ^{*,a,b}	–
Human alveolar adenocarcinoma cells	A549 (CCL-185)	–	149.333 ± 1.528 ^{*,a,c}	19.667 ± 2.082 ^{*,a,b}	–
Human colorectal adenocarcinoma cells	HT-29 (HTB-38)	–	32.000 ± 1.000 ^{*,a,c}	4.000 ± 1.000 ^{*,a,b}	–
Human breast adenocarcinoma cells	MCF-7 (HTB-22)	–	153.333 ± 1.528 ^{*,a,c}	1.667 ± 0.577 ^{*,a,b}	–
Human embryonic kidney 293 cells	HEK-293 (CRL-1573)	–	–	20.667 ± 1.528 ^{*,a,b}	–

* Values are significantly different from negative control group at $p < 0.001$

^{a,b,c} Values are significantly different when compared between samples of each test groups at $p < 0.001$

population. Annexin-V results in Fig. 3 showed that 89.15% of cells were viable in untreated cells, 99.35% of the cells were dead or in the late apoptotic phase in cells treated with doxorubicin and 80.30% of the cells were dead or in the late apoptotic phase in cells treated with crude extracts of (PDA)BL5. The results indicate that both doxorubicin and (PDA)BL5 show apoptotic effects on human prostatic adenocarcinoma cells (PC-3) respectively.

Gas chromatography–mass spectrometry (GC–MS) analysis

Gas chromatography–mass spectrometry (GC–MS) analysis is a technique for analysis and quantitation of organic volatile and semi-volatile compounds and were done on endophytic fungi samples (PDA)BL3 and (PDA)BL5. This is to generally identify the compounds in the samples which may be the possible compounds of interest giving

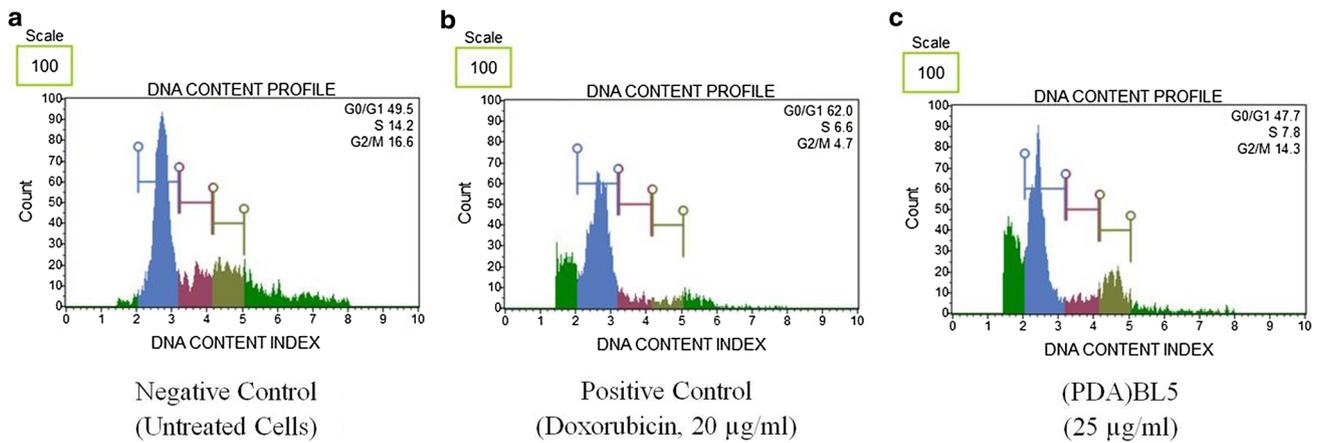


Fig. 2 Cell cycle profile of PC-3 cells untreated and PC-3 cells treated with doxorubicin and (PDA)BL5 determined by flow cytometry analysis using Muse™ Cell Cycle Kit. The figures are from representative experiments carried out at least three times. *Green region* indicates Sub G₀/G₁ phase, *blue region* indicates G₀/G₁ phase,

purple region indicates S phase and *olive region* indicates G₂/M phase. **a** Negative control (Sub G₀/G₁—19.7%), **b** positive control, doxorubicin (Sub G₀/G₁—26.7%) and **c** (PDA)BL5 crude extract (Sub G₀/G₁—30.2%). (Color figure online)

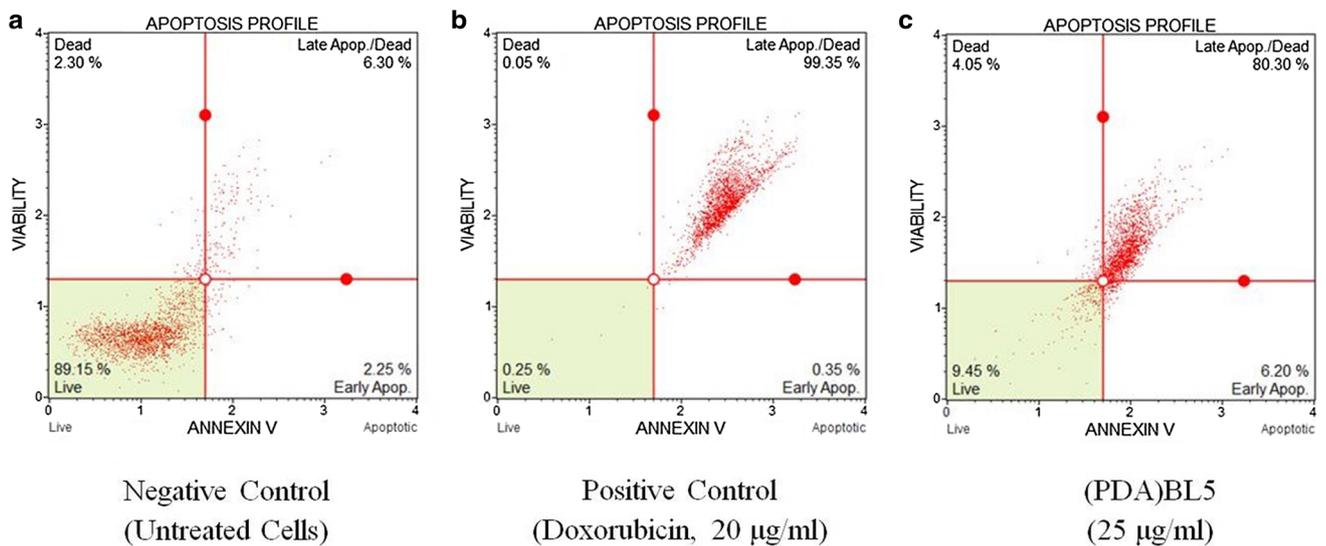


Fig. 3 The percentage of viable, early apoptotic and necrotic/secondary necrotic cells of untreated, doxorubicin-treated and (PDA)BL5-treated PC-3 cells determined by flow cytometry analysis using Muse™ Annexin-V and Dead Cell Kit. The figures are from representative experiments carried out at least three times. The

percentage of viable cells is represented by the *lower left quadrant*; the percentage of early apoptotic and necrotic/secondary necrotic cells is represented by the *lower right* and *upper quadrant*, respectively. **a** Negative control, **b** positive control, doxorubicin and **c** (PDA)BL5 crude extract

the antimicrobial and anticancer properties. A total of 71 volatile metabolites were identified from fungal endophyte sample (PDA)BL3 as shown in Fig. 4 and Table 4. However, only 20 volatile metabolites were identified as volatile metabolites having more than 1% of abundance as highlighted in Table 4. As for fungal endophyte sample (PDA)BL5, a total 54 volatile metabolites were identified as shown in Fig. 5 and Table 5. Where, only 21 volatile metabolites identified were having more than 1% abundance as highlighted in Table 5. However, both GC–MS analysis shows that compound Pyrrolo[1,2-a]pyrazine-1,4-

dione, hexahydro-3-(2-methylpropyl) has the highest abundance at 15.10% abundance at peak 55 for sample (PDA)BL3 and 19.00% abundance at peak 45 for sample (PDA)BL5 respectively. This is followed by Dibutyl phthalate at 13.53% abundance at peak 56, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) at 9.05% abundance at peak 54, Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl at 5.74% abundance at peak 27, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) at 5.53% abundance at peak 47 and many others for sample (PDA)BL3. As for sample (PDA)BL5, it

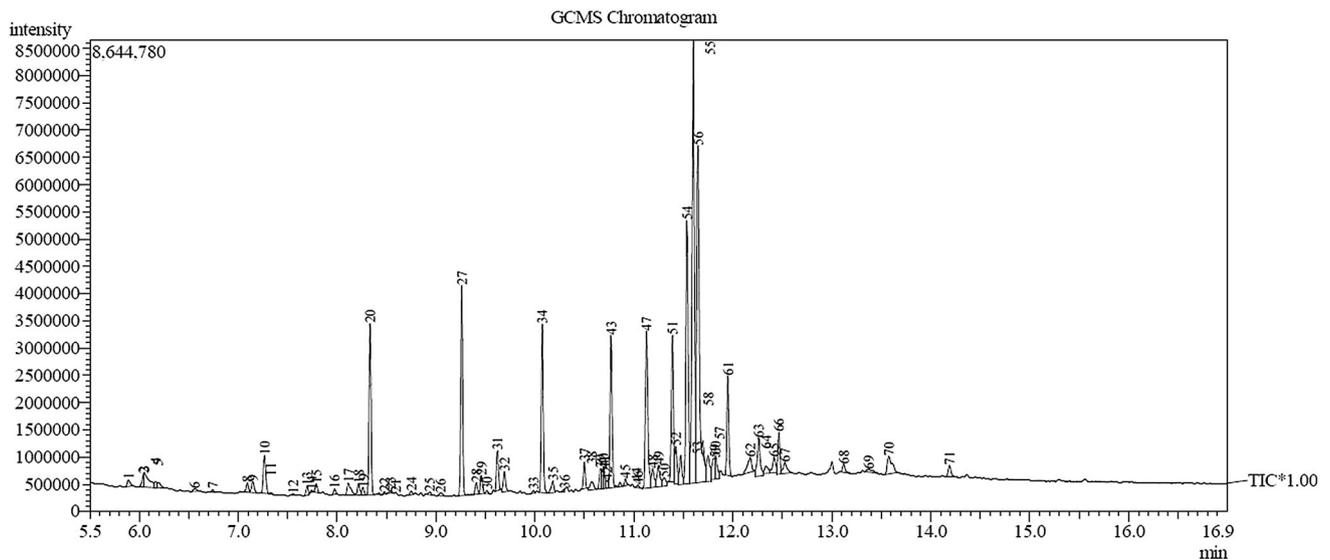


Fig. 4 Chromatogram of gas chromatography coupled with mass spectrometry (GC–MS) of volatile compounds identified from sample (PDA)BL3 isolate using capillary columns with medium polar stationary phases. The 71 identified peaks are shown in Table 4

is followed by Pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) at 12.18% abundance at peak 44, Dibutyl phthalate at 11.88% abundance at peak 46, Pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) at 8.48% abundance at peak 38, 9-Octadecenoic acid, methyl ester, (*E*) at 6.66% abundance at peak 49 and many others. The overlap and none-overlap metabolites identified having more than 1% abundance from sample (PDA)BL3 and (PDA)BL5 are shown in Fig. 6 respectively.

Microscopy

Macroscopic appearance of endophytic fungi isolate (PDA)BL3 and (PDA)BL5 from *Strobilanthes crispus* were taken and are shown in Fig. 7. This is followed by microscopic appearance using normal light microscope to view endophytic fungi stained with lactophenol blue stain shown in Fig. 8 as well as scanning electron microscope view of isolated endophytes of interest in Fig. 9.

Discussion

Endophytic fungi have been recognized as a repository of novel secondary metabolites, some of which have beneficial biological activities potentially used for pharmaceutical, agricultural and biotechnological applications (Bills and Polishook 1991; Strobel and Daisy 2003). The results of the present study agree with these earlier findings. Results show that two endophytic fungi were isolated from *Strobilanthes crispus* having antimicrobial and anticancer

activities. This two endophytic fungi were labeled as (PDA)BL3 and (PDA)BL5. They were successfully isolated using potato dextrose agar medium from the leaves of the medicinal plant.

Molecular identification were used to identify the closely species of interest to the isolated endophytic fungi. Primer of interest used is the 18S rRNA region primers. The 18S rRNA is a nonfunctional RNA sequence located in structural ribosomal RNAs (rRNA). Sequence comparison of the 18S region is widely used in taxonomy and molecular phylogeny because of its high copy number of rRNA genes, which allows easy amplification even from small quantities of DNA, and it possesses a high degree of variation even between closely related species. Sequence obtained from 18S rRNA amplification of (PDA)BL3 and (PDA)BL5 were respectively aligned and further analyzed using BLAST [National Center for Biotechnology Information (NCBI)]. The sequences of the 18S regions were aligned with those of related fungal strains retrieved from the GenBank. Based on phylogenetic tree analysis, results revealed that the isolated endophytic fungi were closely related to 13 genera and 1 class which are *Coniochaeta*, *Lecythophora*, *Phaeoacremonium*, *Togninia*, *Ceratospheeria*, *Ophiostoma*, *Sporothrix*, *Annulusmagnus*, *Ascitendus*, *Rhodoveronaea*, *Lentomitella*, *Barbatospheeria* and *Sordariomycetes*. The phylogenetic tree actually shows that both the sample (PDA)BL3 and (PDA)BL5 were most closely related to the *Sordariomycetes* sp. *Sordariomycetes* have been reported to be the most abundant endophytic groups isolated from various plant families (Supaphon et al. 2014). This indicates that it is possible to isolate these two endophytic fungi of interests

Table 4 71 volatile metabolites identified from sample (PDA)BL3 using dichloromethane solvent via gas chromatography coupled with mass spectrometry (GC-MS)

Peak	Compound name	Molecular formula	RT (min)	MW	Abundance (%)
1	2-Heptenal, (Z)	C7H12O	5.891	112	0.29
2	Pentanoic acid, 3-hydroxy-ethyl ester	C7H14O3	6.042	146	0.31
3 ^a	Cyclotetrasiloxane, octamethyl	C8H24O4Si4	6.050	296	1.10
4	Silane, [3-(2,3-epoxypropoxy) propyl]ethoxydimethyl	C10H22O3Si	6.166	218	0.23
5	Furan, 2-pentyl	C9H14O	6.192	138	0.22
6	1-(4-Hydroxy-3-methoxyphenyl)-1-ethoxyacetic acid ethyl ester, <i>O</i> -trimethylsilyl	C16H26O5Si	6.560	326	0.14
7	Decane, 3,7-dimethyl	C12H26	6.742	170	0.06
8	Undecane	C11H24	7.093	156	0.31
9	Nonanal	C9H18O	7.147	142	0.42
10 ^a	Cyclopentasiloxane, decamethyl	C10H30O5Si5	7.266	370	1.36
11	4-Piperidinone, 2,2,6,6-tetramethyl	C9H17NO	7.332	155	0.04
12	2-Nonenal, (E)	C9H16O	7.553	140	0.05
13	Silane, dimethyl(2-methoxyethoxy)hexyloxy	C11H26O3Si	7.696	234	0.31
14	2-[di(<i>tert</i> -Butyl)silyloxymethyl] tetrahydrofuran	C13H28O2Si	7.742	244	0.06
15	Allyloxydi(<i>tert</i> -butyl)silane	C11H24OSi	7.791	200	0.27
16	Tridecanoic acid, 3-hydroxy-, ethyl ester	C15H30O3	7.975	258	0.17
17	Silane, trimethyl(1-methylbutoxy)	C8H20OSi	8.112	160	0.69
18	2-Decenal, (E)	C10H18O	8.217	154	0.41
19	Nonane, 5-methyl-5-propyl	C13H28	8.267	184	0.24
20 ^a	Cyclohexasiloxane, dodecamethyl	C12H36O6Si6	8.333	444	4.70
21	2,4-Dodecadienal, (E,E)	C12H20O	8.423	180	0.04
22	Phenol, <i>m-tert</i> -butyl	C10H14O	8.480	150	0.07
23	Dodecane, 4,6-dimethyl	C14H30	8.539	198	0.06
24	1,8-Naphthalenedione, 8a-ethylperhydro	C12H18O2	8.750	194	0.12
25	1-Dodecanol	C12H26O	8.935	186	0.10
26	Benzene, 1-[2-(2-chloroethoxy)ethoxy]-4-(1,1,3,3-tetramethylbutyl)	C18H29ClO2	9.045	312	0.13
27 ^a	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl	C12H38O5Si6	9.260	430	5.74
28	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)	C14H20O2	9.407	220	0.38
29	Eicosane	C20H42	9.460	282	0.56
30	Pentadecane	C15H32	9.515	212	0.08
31 ^a	Phenol, 2,4-bis(1,1-dimethylethyl)	C14H22O	9.621	206	1.21
32	2,6-Dimethyltridecanenitrile	C15H29 N	9.691	223	0.67
33	<i>n</i> -Pentadecanol	C15H32O	9.984	228	0.07
34 ^a	Cyclooctasiloxane, hexadecamethyl	C16H48O8Si8	10.074	592	4.73
35	3,4-Xylyl 3,5-di- <i>tert</i> -butylbenzoate	C23H30O2	10.181	338	0.48
36	2,5-Piperazinedione, 3-methyl-6-(1-methylethyl)	C8H14N2O2	10.300	170	0.08
37	Eicosane	C20H42	10.499	282	0.74
38	2,5-di- <i>O</i> -Acetyl-3,4,6-tri- <i>O</i> -methyl- β -gluconitrile	C13H21NO7	10.574	303	0.44
39	Uric acid	C5H4N4O3	10.664	168	0.72
40	3,6-Nonadecadione	C19H36O2	10.692	296	0.41
41	Decane, 1-iodo	C10H21I	10.700	268	0.38
42	DL-Alanyl-L-leucine	C9H18N2O3	10.730	202	0.21
43 ^a	Cyclononasiloxane, octadecamethyl	C18H54O9Si9	10.770	666	4.78
44	3,5-di- <i>tert</i> -Butyl-4-hydroxybenzaldehyde	C15H22O2	10.858	234	0.16
45	<i>n</i> -Pentadecanol	C15H32O	10.914	228	0.21
46	Tetradecanal	C14H28O	11.040	212	0.08

Table 4 continued

Peak	Compound name	Molecular formula	RT (min)	MW	Abundance (%)
47 ^a	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	C11H18N2O2	11.128	210	5.53
48	3,6-Diisopropylpiperazin-2,5-dione	C10H18N2O2	11.192	198	0.78
49 ^a	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	C11H18N2O2	11.248	210	1.01
50	1-Nonadecene	C19H38	11.308	266	0.40
51 ^a	Cyclononasiloxane, octadecamethyl	C18H54O9Si9	11.389	666	4.55
52 ^a	Tetracontane	C40H82	11.424	562	1.21
53 ^a	Hexadecanoic acid, methyl ester	C17H34O2	11.473	270	1.04
54 ^a	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	C11H18N2O2	11.536	210	9.05
55 ^a	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	C11H18N2O2	11.600	210	15.10
56 ^a	Dibutyl phthalate	C16H22O4	11.647	278	13.53
57	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a;1',2'-d]pyrazine	C14H22N2O2	11.697	250	0.23
58 ^a	Hexadecanoic acid, ethyl ester	C18H36O2	11.750	284	1.35
59	2H-Pyrrol-2-one, 1,5-dihydro-4-(1-pyrrolidinyl)	C8H12N2O	11.817	152	0.80
60	4(3H)-Pyrimidinone, 3-ethyl-2,6-dimethyl	C8H12N2O	11.825	152	0.82
61 ^a	Benzoic acid, 2,6-bis[(trimethylsilyloxy)-, trimethylsilyl ester	C16H30O4Si3	11.950	370	2.72
62	9-Octadecenoic acid, methyl ester, (E)	C19H36O2	12.176	296	0.90
63 ^a	Octadecanoic acid, methyl ester	C19H38O2	12.262	298	1.74
64	cis-9-Hexadecenal	C16H30O	12.336	238	0.39
65	Octadecanoic acid	C18H36O2	12.417	284	0.70
66 ^a	Cyclononasiloxane, octadecamethyl	C18H54O9Si9	12.465	666	1.31
67	12-Oxododecanoic acid, ethyl ester	C14H26O3	12.526	242	0.62
68	Tetrapentacontane	C54H110	13.120	758	0.31
69	Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'.alpha.,10.alpha.)	C33H37N5O5	13.372	583	0.23
70 ^a	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)	C14H16N2O2	13.577	244	1.22
71	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	C16H22O4	14.190	278	0.47

RT retention time, MW molecular weight

^a 20 volatile metabolites identified having more than 1% abundance from sample (PDA)BL3 via gas chromatography coupled with mass spectrometry (GC-MS)

which are most closely related to *Sordariomycetes* sp. from *Strobilanthes crispus* that have promising antimicrobial and anticancer properties. *Sordariomycetes* is one of the largest classes of fungi in the division *Ascomycota* and subdivision *Pezizomycotina* consisting of 28 orders, 90 families and 1344 genera (Maharachchikumbura et al. 2015).

Antimicrobial activities were screened against 8 g-positive bacteria and 16 g-negative bacteria by dichloromethane extract suspensions or secondary metabolites of (PDA)BL3 and (PDA)BL5. These 24 bacteria tested are among the pathogenic bacteria generally affecting the health of mankind which are available in our laboratory. For that this preliminary screening of crude extracts is the hope to obtain potential antimicrobial properties in endophytes. With that, results show that (PDA)BL3 has better antimicrobial activity as compared (PDA)BL5. Sample (PDA)BL3 expressed antimicrobial activity against 6 g

positive bacteria which are *Bacillus cereus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus saprophyticus* as compared to sample (PDA)BL5 not expressing any antimicrobial activity towards all 24 bacteria.

On the other hand, anticancer activities were screened against 5 cancer cell lines and 1 normal cell line by dichloromethane extract suspensions or secondary metabolites of the isolated endophytic fungi sample (PDA)BL3 and (PDA)BL5. Results show that (PDA)BL5 has better anticancer activity as compared (PDA)BL3. Sample (PDA)BL5 expressed anticancer activity towards all 5 cancer cell lines which are human prostatic adenocarcinoma cells (PC-3), human hepatocellular carcinoma cell (HEPG2), human alveolar adenocarcinoma cells (A549), human colorectal adenocarcinoma cells (HT-29), and human breast adenocarcinoma cells (MCF-7) as

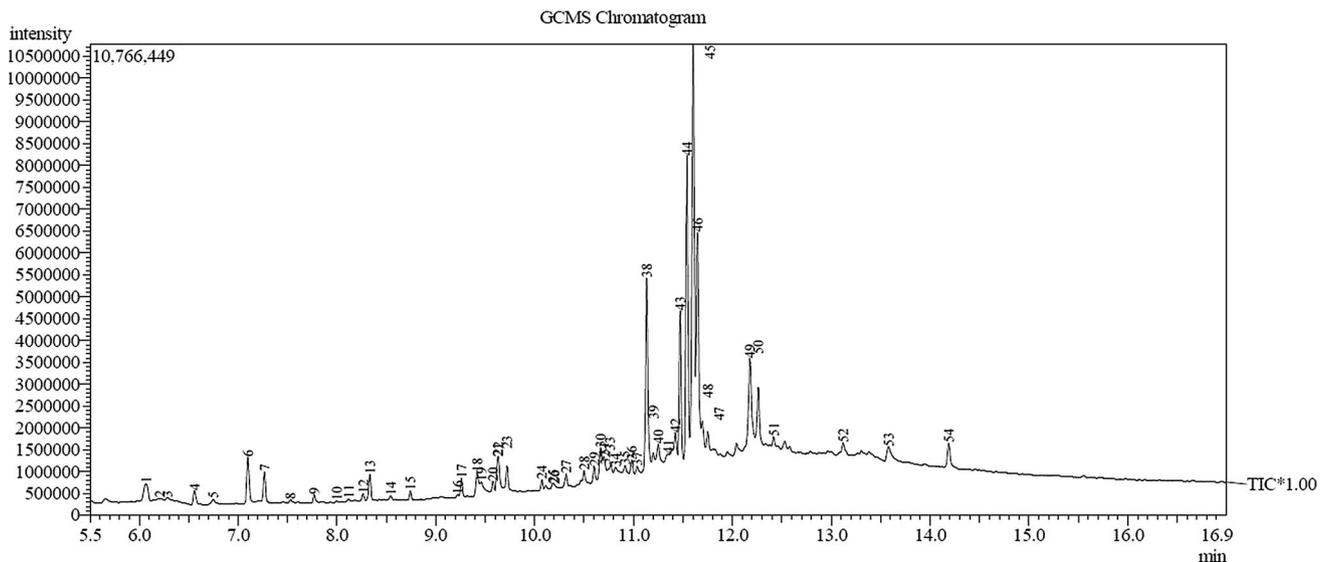


Fig. 5 Chromatogram of gas chromatography coupled with mass spectrometry (GC–MS) of volatile compounds identified from sample (PDA)BL5 isolate using capillary columns with medium polar stationary phases. The 54 identified peaks are shown in Table 5

compared to sample (PDA)BL3 having anticancer activity to 2 cancer cell lines which are human prostatic adenocarcinoma cells (PC-3) and human hepatocellular carcinoma cell (HEPG2) at a higher dose concentration. Results show that crude extract sample (PDA)BL5 showed anticancer activity towards all 5 cancer cell lines. These findings may not appear to contain any targeted therapy but exploit an Achilles' heel common to most cancer cells in a manner similar to chemotherapy. This is because the main aim of this research to undergo a preliminary study of potential endophytes having compound for anticancer properties. Thus for that, further in-depth tests on its pathway of individual cancer properties of different cancer cell line will be done in the near future. Besides that, sample (PDA)BL5 did not show significant difference to positive control for anticancer test towards human prostatic adenocarcinoma cells (PC-3). This indicates that (PDA)BL5 has a rather similar IC_{50} concentration as the positive control, doxorubicin against prostate cancer cells. This points out that (PDA)BL5 can be a potential crude extract for treatment against prostate cancer cells. However, much further tests have to be done.

For that, flow cytometry analysis of cell cycle and Annexin-V tests were done on crude extract sample of (PDA)BL5 towards human prostatic adenocarcinoma cells, PC-3. This is because cytotoxicity activity of (PDA)BL5 was found to be highly effective towards PC-3 as compared to the other cancer cell lines. In cell cycle study as shown in Fig. 2, significant hypodiploid DNA content peaks were observed in Sub G_0/G_1 phase in a PC-3 cells treated with doxorubicin at 26.7% and sample (PDA)BL5 at 30.2%. This phase indicates the presence of apoptotic population

or apoptosis occurrence and hence, further validation can be done via Annexin-V tests. Previous studies have shown that presence of this Sub G_0/G_1 population is due to breakage of the linkers between the nucleosomes in the chromatin by endonucleases (Darzynkiewicz et al. 1992; Hanon et al. 1996; Vanparys et al. 2006; Ooi et al. 2015). Results also show that sample (PDA)BL5 may have transient arrest at G_2/M phase. However further studies need to be done to validate this finding. In Annexin-V study as shown in Fig. 3, PC-3 untreated cells revealed 89.15% of viable cells; doxorubicin-treated PC-3 cells showed 99.35% of the cells were dead or in the late apoptotic phase and (PDA)BL5-treated PC-3 cells showed 80.30% of the cells were dead or in the late apoptotic phase in cells treated with crude extracts of (PDA)BL5. These results indicate that doxorubicin and (PDA)BL5 can potently inhibit cell viability of human prostatic adenocarcinoma cells (PC-3) (Hsieh et al. 2014; Ooi et al. 2015). Nevertheless, further tests on the possible pathways such as the caspase pathways needs to be done in the near future.

Apart from that, it is interesting to find out that even though these two endophytic fungi samples maybe of similar species, they have very different biological activities where (PDA)BL3 has better antimicrobial activity and (PDA)BL5 having better anticancer activity. The different effects may be due to the possible synergistic effect of the compounds in the crude extract in each of the isolated endophytic fungi giving positive and negative antimicrobial and anticancer properties (Lang et al. 2005; Goutam et al. 2016).

Thus, for that gas chromatography mass spectrometry (GC–MS) analysis were done on 2 samples which are

Table 5 54 volatile metabolites identified from sample (PDA)BL5 using dichloromethane solvent via gas chromatography coupled with mass spectrometry (GC-MS)

Peak	Compound name	Molecular formula	RT (min)	MW	Abundance (%)
1 ^a	Cyclotetrasiloxane, octamethyl	C ₈ H ₂₄ O ₄ Si ₄	6.067	296	1.39
2	1-Butyl(dimethyl)silyloxypropane	C ₉ H ₂₂ O ₂ Si	6.206	174	0.17
3	Undecane	C ₁₁ H ₂₄	6.285	156	0.14
4	1-(4-Hydroxy-3-methoxyphenyl)-1-ethoxyacetic acid ethyl ester	C ₁₆ H ₂₆ O ₅ Si	6.559	326	0.71
5	Dodecane, 4,6-dimethyl	C ₁₄ H ₃₀	6.747	198	0.35
6 ^a	Undecane	C ₁₁ H ₂₄	7.098	156	2.23
7 ^a	Cyclopentasiloxane, decamethyl	C ₁₀ H ₃₀ O ₅ Si ₅	7.266	370	1.46
8	Benzene, 4-chloro-1,2-dimethyl	C ₈ H ₉ Cl	7.529	140	0.13
9	Octadecanoic acid, 2-[(trimethylsilyl)oxy], methyl ester	C ₂₂ H ₄₆ O ₃ Si	7.769	386	0.35
10	Benzaldehyde, 2,4-dimethyl	C ₉ H ₁₀ O	7.997	134	0.06
11	2-Coumaranone	C ₈ H ₆ O ₂	8.117	134	0.09
12	Dodecane, 4,6-dimethyl	C ₁₄ H ₃₀	8.264	198	0.30
13 ^a	1,3,5-Tris(trimethylsilyloxy) benzene	C ₁₅ H ₃₀ O ₃ Si ₃	8.333	342	1.04
14	Tridecanoic acid, 4,8,12-trimethyl-, methyl ester	C ₁₇ H ₃₄ O ₂	8.543	270	0.19
15	(4-Hydroxy-3-methoxyphenyl) ethylene glycol tris(trimethylsilyl) ether	C ₁₈ H ₃₆ O ₄ Si ₃	8.742	400	0.33
16	2-Allyl-4-methylphenol	C ₁₀ H ₁₂ O	9.221	148	0.07
17	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]	C ₁₂ H ₃₆ O ₄ Si ₅	9.259	384	0.62
18 ^a	Acetophenone, 4'-hydroxy	C ₈ H ₈ O ₂	9.418	136	1.31
19	Heptadecane	C ₁₇ H ₃₆	9.459	240	0.72
20	Acetic acid, [bis(trimethylsilyl)oxy]phosphinyl], trimethylsilyl ester	C ₁₁ H ₂₉ O ₅ PSi ₃	9.578	356	0.35
21	Phenol, 2,4-bis(1,1-dimethylethyl)	C ₁₄ H ₂₂ O	9.625	206	0.93
22 ^a	Dodecanoic acid, methyl ester	C ₁₃ H ₂₆ O ₂	9.629	214	1.15
23 ^a	Benzene, 1-methyl-4-(1,2,2-trimethylcyclopentyl), (R)	C ₁₅ H ₂₂	9.720	202	1.18
24	Benzoic acid, 2,6-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	C ₁₆ H ₃₀ O ₄ Si ₃	10.073	370	0.35
25	4-Amino-7-diethylamino-chromen-2-one	C ₁₃ H ₁₆ N ₂ O ₂	10.187	232	0.25
26	Tetrahydrofuran-2-one, 5-[1-hydroxyhexyl]	C ₁₀ H ₁₈ O ₃	10.200	186	0.08
27	4-Amino-5-imidazole carboxamide, <i>N,N,O</i> -tris(trimethylsilyl)	C ₁₃ H ₃₀ N ₄ O ₅ Si ₃	10.318	342	0.54
28	Eicosane	C ₂₀ H ₄₂	10.500	282	0.35
29	Methyl tetradecanoate	C ₁₅ H ₃₀ O ₂	10.602	242	0.61
30 ^a	Uric acid	C ₅ H ₄ N ₄ O ₃	10.669	168	1.38
31	1,3-Dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone	C ₆ H ₁₂ N ₂ O	10.700	128	0.77
32	Cyclopentanone, 3,3,4-trimethyl-4-(4-methylphenyl)	C ₁₅ H ₂₀ O	10.717	216	0.13
33	3,6-Nonadecadione	C ₁₉ H ₃₆ O ₂	10.758	296	0.08
34	3,3,4-Trimethyl-4-(4-methylphenyl)cyclopentanol	C ₁₅ H ₂₂ O	10.818	218	0.23
35	1-Nonadecene	C ₁₉ H ₃₈	10.914	266	0.23
36	Benzoic acid, 2,6-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	C ₁₆ H ₃₀ O ₄ Si ₃	10.982	370	0.53
37	Isopropyl myristate	C ₁₇ H ₃₄ O ₂	11.037	270	0.33
38 ^a	Pyrrrolo[1,2- <i>a</i>]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	C ₁₁ H ₁₈ N ₂ O ₂	11.134	210	8.48
39	Piperazine-3,5-dione, 1-tetradecanoyl	C ₁₈ H ₃₂ N ₂ O ₃	11.200	324	0.81
40 ^a	Pyrrrolo[1,2- <i>a</i>]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	C ₁₁ H ₁₈ N ₂ O ₂	11.251	210	2.45
41 ^a	1,3-Benzenediol, <i>o</i> -ethoxycarbonyl	C ₉ H ₁₀ O ₄	11.348	182	1.12
42 ^a	Eicosane	C ₂₀ H ₄₂	11.424	282	1.42
43 ^a	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	11.473	270	5.62
44 ^a	Pyrrrolo[1,2- <i>a</i>]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	C ₁₁ H ₁₈ N ₂ O ₂	11.540	210	12.18
45 ^a	Pyrrrolo[1,2- <i>a</i>]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	C ₁₁ H ₁₈ N ₂ O ₂	11.603	210	19.00
46 ^a	Dibutyl phthalate	C ₁₆ H ₂₂ O ₄	11.647	278	11.88
47 ^a	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2- <i>a</i> ;1',2'- <i>d</i>]pyrazine	C ₁₄ H ₂₂ N ₂ O ₂	11.698	250	1.36
48	Behenic alcohol	C ₂₂ H ₄₆ O	11.751	326	0.85

Table 5 continued

Peak	Compound name	Molecular formula	RT (min)	MW	Abundance (%)
49 ^a	9-Octadecenoic acid, methyl ester, (E)	C19H36O2	12.178	296	6.66
50 ^a	Octadecanoic acid, methyl ester	C19H38O2	12.262	298	3.87
51	Eicasone	C20H42	12.418	282	0.33
52	Eicasone	C20H42	13.120	282	0.55
53 ^a	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)	C14H16N2O2	13.580	244	1.04
54 ^a	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	C16H22O4	14.189	278	1.25

RT retention time, MW molecular weight

^a 21 volatile metabolites identified having more than 1% abundance from sample (PDA)BL5 via gas chromatography coupled with mass spectrometry (GC–MS)

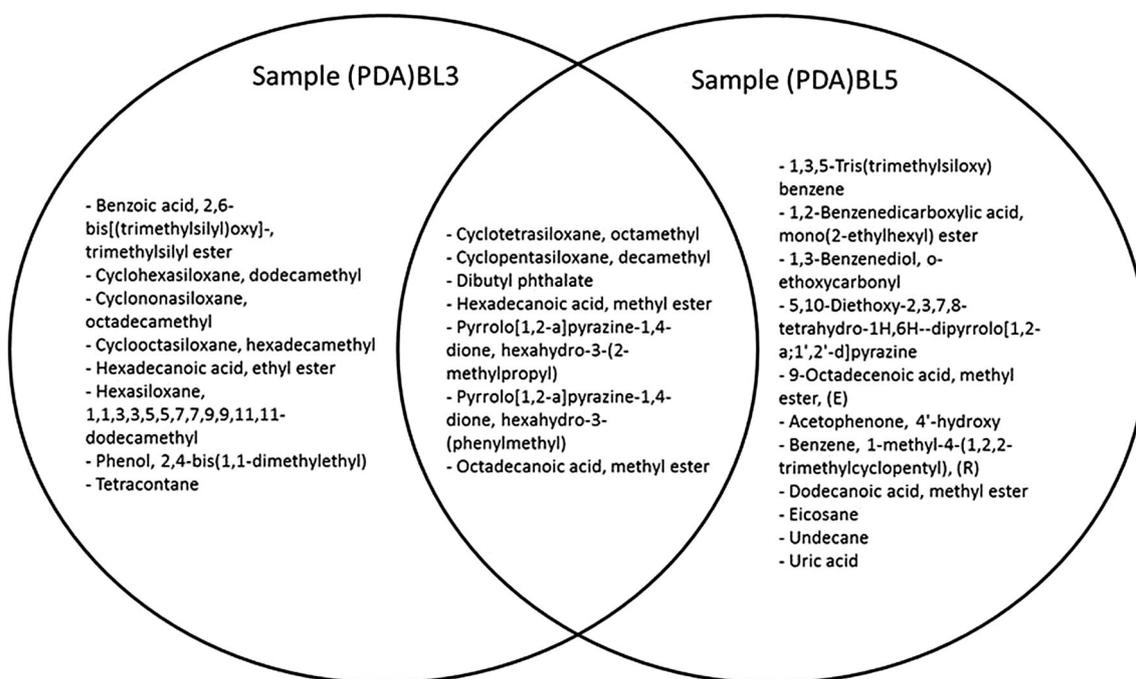


Fig. 6 Overlap and none-overlap metabolites identified having more than 1% abundance from sample (PDA)BL3 and (PDA)BL5

(PDA)BL3 that has highest significant antimicrobial activity against 6 bacteria and (PDA)BL5 that has highest significant anticancer activity against all 5 cancer cell lines and not showing any toxicity in the normal cell line. This is to generally know the compounds in the samples which could be the possible compounds of interest giving the antimicrobial and anticancer properties. A total of 71 volatile metabolites were identified from sample (PDA)BL3 using dichloromethane solvent via gas chromatography coupled with mass spectrometry (GC–MS) analysis and a total of 54 volatile metabolites were identified from sample (PDA)BL5 respectively. However, only a total of 20 volatile metabolites identified from sample

(PDA)BL3 and 21 volatile metabolites identified from sample (PDA)BL5 having more than 1% abundance. The other volatile metabolites are not considered because their abundance may be too little to be the effect for the antimicrobial and anticancer properties (Siddiquee et al. 2015). However, both GC–MS analysis shows that compound Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) has the highest abundance at 15.10% abundance for sample (PDA)BL3 and 19.00% abundance for sample (PDA)BL5 respectively despite the two samples having rather different biological activity. Previous studies have shown that this particular compound of interest was also isolated from an endophytic fungi isolated from moss

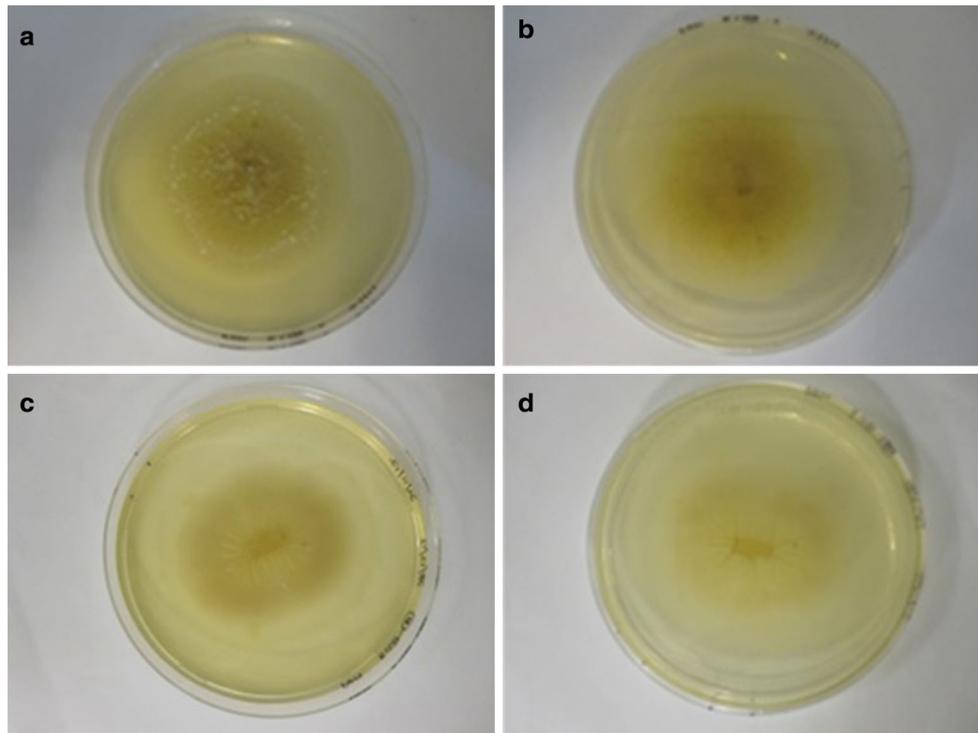


Fig. 7 Macroscopic appearance of endophytic fungi isolated from *Strobilanthes crispus*. **a** Front view of agar plate (PDA)BL3, **b** back view of agar plate (PDA)BL3, **c** front view of agar plate (PDA)BL5 and **d** back view of agar plate (PDA)BL5

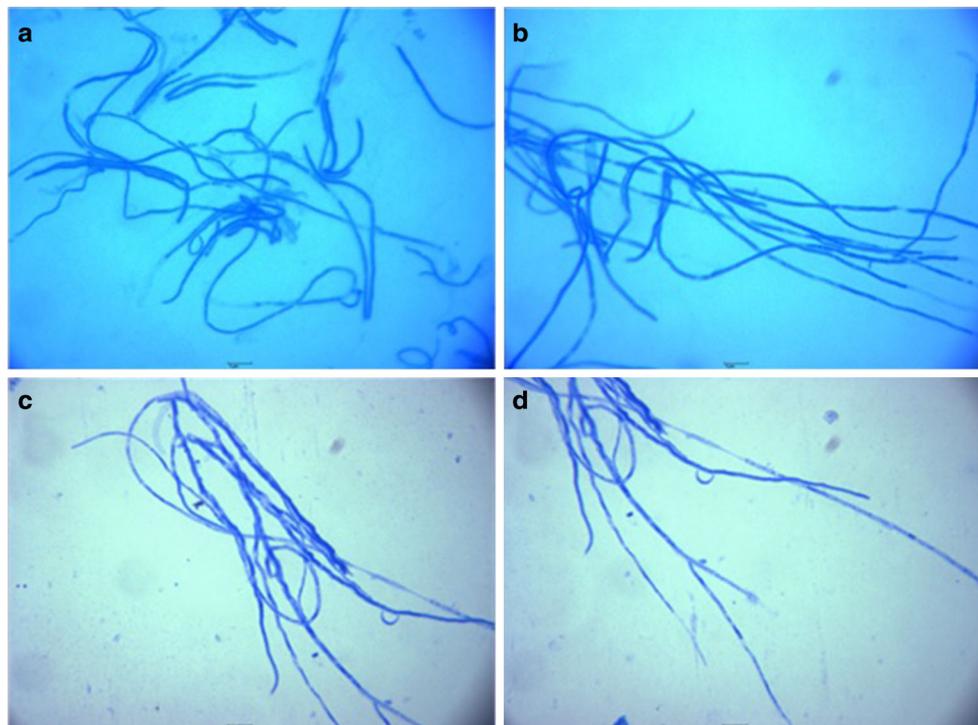


Fig. 8 Microscopic appearance of endophytic fungi isolated from *Strobilanthes crispus* stained with lactophenol cotton blue stain. **a**, **b** Microscopic hyphae appearance ($\times 100$) of (PDA)BL3. **c**, **d** microscopic hyphae appearance ($\times 100$) of (PDA)BL5

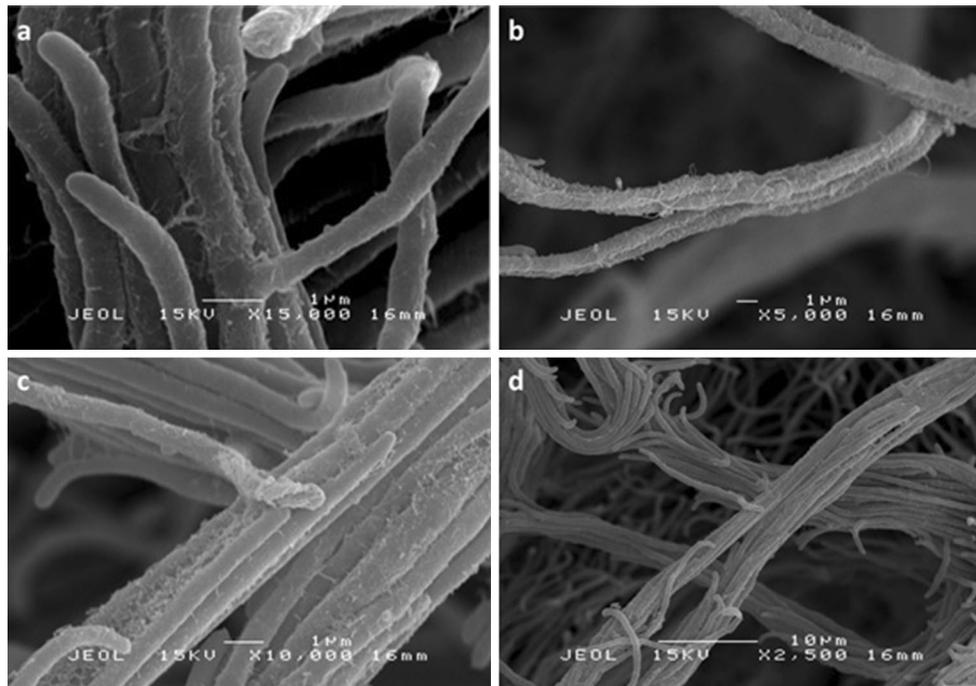


Fig. 9 Scanning electron micrographs of endophytic fungi isolated from *Strobilanthes crispus* using Joel JSM-6400 SEM at 15 kV. **a** Scanning electron micrograph of hyphae appearance ($\times 15000$) of (PDA)BL3, **b** scanning electron micrograph of hyphae appearance

($\times 5000$) of (PDA)BL3, **c** scanning electron micrograph of hyphae appearance ($\times 10000$) of (PDA)BL5 and **d** scanning electron micrograph of hyphae appearance ($\times 2500$) of (PDA)BL5

Schistidium antarctici, a plant found in Antarctica having antimicrobial activity (Melo et al. 2014).

The possibility of two closely related fungal endophyte samples having different biological effects could be due to its synergistic effect of the compounds in the crude extract exhibiting its antimicrobial and anticancer activities respectively despite having high abundance of the same compound of interest. Results show that sample (PDA)BL3 contains other compounds at rather high abundance which is Dibutyl phthalate at 13.10% abundance. Previous studies have proven that Dibutyl phthalate has high antimicrobial efficacy showing wide spectra of antimicrobial activity (Roy et al. 2006; Khatiwora et al. 2012). In comparison between sample (PDA)BL3 and (PDA)BL5, (PDA)BL3 has higher abundance of Dibutyl phthalate. This may indicate the synergistic effect of the few compounds in the crude extract of (PDA)BL3 containing high abundance of Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methyl-propyl) and Dibutyl phthalate could be the cause of the different biological effects with (PDA)BL5. Apart from that, in sample (PDA)BL5 there are also presence of 9-Octadecenoic acid, methyl ester which is a type of fatty acid methyl ester found to be isolated from medicinal plants which are traditionally used to treat cancer (Gopal et al. 2013). The synergistic effects of the various major compounds in the crude extract could be the cause of the different biological effect. Studies have also shown that

synergistic effects do play a vital role in showing anti-properties in many researches done these days (Koech et al. 2013; Abubakar et al. 2016; Albano et al. 2016). There is clear evidence that the synergistic studies can be a good source of bioactive substances that could possess broad spectrum activity. However, further additional analysis is required to investigate which properties are significantly correlated among these active secondary metabolites in the isolated endophytic fungi.

These findings as far as I have reported do not mark the end of this finding. They are many future directions ahead. The future directions would be to further identify and isolate the compounds of interest for antimicrobial and anticancer screening via Preparative High Performance Liquid Chromatography (HPLC) and reconfirm the structure using Nuclear magnetic resonance (NMR) and how the compounds may react synergistically with one another to produce the antimicrobial and anticancer properties. As well as, further studies of the pathways involved in the antimicrobial and anticancer properties.

This study shows that fungal endophytes isolated from medicinal plant, *Strobilanthes crispus* showed promising possible sources of antimicrobial and anticancer compounds. This may have further verified that fungi namely the endophytes, which reside within medicinally important plants, may have acquired their medicinal abilities from the host plant whereby *Strobilanthes crispus* was traditionally

known to have been able to treat cancer and have antimicrobial properties. Therefore, this work has further indicated the medicinal and industrial potential of fungal endophytes.

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