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Antimicrobial and Cytotoxic Activities of A compound Produced by An Endophytic Fungus Isolated from The Leaves of *Coleus amboinicus* Lour

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ABSTRACT

Various compounds are produced by endophytes and possess antimicrobial activities. An endophytic fungus originated from *Coleus amboinicus* was discovered to produce compounds having antimicrobial activities. This study was intended to explore the bioactive compounds responsible for antibacterial activities and possibly also for their potential as anticancer agents. Isolation of the bioactive compounds was conducted by bioassay-guided fractionation using disc diffusion method for antimicrobial screening activities. The antimicrobial potential was determined by IC_{50} and MBC (Minimum Bactericidal Concentration) values. Cytotoxicity testing was conducted by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay against T47D breast cancer cell line and normal Vero cells. Structure elucidation of the bioactive compound was done by mean of spectroscopic data. Disc diffusion test of four fractions followed by examination of IC_{50} and MBC values of isolated compounds showed that a compound within fraction 2 was found to be the most active one with IC_{50} of 0.73, 1.24, 1.17, 0.31 and 1.27 μ g/ml against *S. aureus*, *B. subtilis*, *E. coli*, *S. mutans*, and *P. aeruginosa* respectively. This compound also killed T47D (IC_{50} of 174 μ g/ml) and Vero cells (IC_{50} of 214 μ g/ml). The bioactive compound was suggested to be an aromatic compound having methoxy, hydroxyl and methyl groups. These data demonstrated the potential of an endophytic fungus *Athelia rolfsii* as a producer of antimicrobial agents, yet its potential for producing anticancer agents still need to be explored.

Keywords: antimicrobial activity, *Athelia rolfsii*, *Coleus amboinicus*, cytotoxicity, endophytic fungi

INTRODUCTION

Whilst many reported the presence of bioactive compounds isolated from plants, there was an increasing interest of exploring endophytes as source of potential lead compounds [1-6]. Various endophytic fungi originated from medicinal plants synthesized bioactive compounds having pharmaceutical importance with some metabolites were also present in the host plants. For example, Taxol produced by *Taxomyces andreanae* was initially found in the host plant *Taxus brevifolia*, whilst Camptothecin, an anti-cancer drug originally isolated from *Camptotheca acuminata*, had been reported to be manufactured by its endophytic fungus [7,8]. Various compounds produced by endophytes had also been reported to have antimicrobial activities [9]; 3-Hydroxypropionic acid produced by

Laguncularia racemosa was also synthesized by an endophytic fungus *Diaporthe phaseolorum* [10]. Piperin commonly found in *Piper longum* Lour was also produced by an endophytic fungus *Periconia* sp. and showed to be active against *Mycobacterium tuberculosis* dan *Mycobacterium smegmatis* [11]. Alkaloids compounds within *Bauhinia guianensis* medicinal plant, were also synthesized by an endophytic fungus *Aspergillus* sp. EJC08 and were found to be have antibacterial properties [12]. Endophytic fungi of *Azadirachta indica* were found to synthesize various bioactive compounds having antimicrobial potencies [13]. Similarly, Wu et al. had isolated saponin-producing fungal endophytes of *Aralia elata* which possessed antimicrobial activity [14].

9 *Coleus amboinicus* Lour is a medicinal plant used traditionally to treat various diseases such as urinary tract infection, digestive disorder, malaria, cough, asthma and fever [15]. Many phytochemicals had been isolated from *C. amboinicus* including terpenoids, phenolics and flavonoids which were reported to exhibit pharmacological and nutritional values [16,17]. Essential oil of this plant and its major component carvacrol, for example, was shown to inhibit the growth of multidrug resultant *S. aureus* [18]. Various solvent extracts, fractions as well as nanoparticles of *C. amboinicus* exhibited antibacterial activities [16]. Despite its medicinal uses as antimicrobial agents, *C. amboinicus* also had potential as antitumor. Ethanolic extract nanoparticles of this plant was reported to inhibit the growth of T47D cell lines [19], whilst its aqueous extracts at a dose of 200 mg / kg was able to inhibit the growth of Ehrlich ascites carcinoma [20]. The fact that cancer incidence and mortality were increasing worldwide [21] and there was high rate of resistance towards antibiotics [22], it is of interest to explore the potential of endophytic fungi in producing compounds having potential as anti-cancer and antimicrobial agents. Previous study reported an endophytic fungus from *C. amboinicus* which produced compounds having antimicrobial activities. This study was aimed to characterize bioactive compound responsible for the antimicrobial activities and explored its potential for cytotoxicity studies.

MATERIALS AND METHODS

Materials

Silica gel F₂₅₄ (Merck, Darmstadt, Germany), Silica gel 60 PF₂₅₄ containing gypsum (Merck, Darmstadt, Germany), Dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany), methanol (Merck, Darmstadt, Germany), chloroform (Merck, Darmstadt, Germany), n-hexane (Merck, Darmstadt, Germany), ethyl acetate (Merck, Darmstadt, Germany). Potato Dextrose Agar (PDA) (Sigma-Aldrich, Missouri, USA), Dextrose (Sigma-Aldrich, Missouri, USA), Nutrient Agar (NA) (Sigma-Aldrich, Missouri, USA), Mueller Hinton (Oxoid, Massachusetts, USA). A fungal strain identified as *Athelia rolfsii* was isolated from *C. amboinicus*. For routine culture, the isolated fungus was inoculated on PDA plates without antibiotics and the cultures were collected at the Pharmaceutical Biology Department, Faculty of Pharmacy Universitas Gadjah Mada, Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Massachusetts, USA), Fetal Bovine Serum (FBS) (Gibco, Massachusetts, USA), Penicillin-

Streptomycin (Gibco, Massachusetts, USA), Fungizone (Gibco, Massachusetts, USA), Sodium bicarbonate (Gibco, Massachusetts, USA), L-glutamine (Gibco, Massachusetts, USA). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Invitrogen, Massachusetts, USA) and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, Missouri, USA).

Isolation of endophytic fungus

The fungal strain was obtained from the leaves of *C. amboinicus* collected from Medicinal Plant Garden, Faculty of Pharmacy, Universitas Gadjah Mada using modified protocol described by Ding et al. [23]. The plant materials was identified by Dr. Djoko Santosa, M.Si with a voucher specimen number 253 was deposited for reference in the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada. Briefly, after 10 minutes rinsing with running tap water the leaves were treated with surface disinfection using 70% ethanol for 1 minute. They were subsequently immersed on 5% sodium hypochlorite for 3 minutes, drained and re-soaked in 70% ethanol for 30 seconds to accomplish sufficient surface sterilisation. The leaves were thoroughly washed in sterile distilled water thrice. Small cuts (1 cm long segments) of surface sterilized leaves were placed onto petri dish containing PDA medium supplemented with 30 µg/ml streptomycin. The PDA plates were incubated at 25°C until the hyphal tips grew out from the segments. The individual hyphal tips were removed and transferred into new PDA plates containing 30 µg/ml streptomycin. Following 10 – 14 days of incubation, the pure culture was obtained by several passaging and subsequent growth on PDA plates without antibiotics.

Identification of endophytic fungus

The endophytic fungal strain was identified based on Internal Transcribed Spacer (ITS) partial genetic analysis of the fungal ribosomal DNA with primers ITS4: 5'-TCCTCCGCTTAT TGATAT GC - ITS 3' and Primary 5: 5' - AAAAGTAGTCGTGGAAACAAG G -3 [24,25]. The fungal DNA was extracted using nucleon PHYTO pure reagents and the amplified product was purified using polyethylene glycol (PEG) precipitation method [26], followed by cycle sequencing. The purified DNA was sequenced by automated DNA sequencer and analyzed using bioedit program (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). The DNA sequence was submitted for homology

analysis to GenBank using BLAST program. The ribosomal gene data base DDBJ (<http://blast.ddbj.nig.ac.jp/>) or NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to identify the fungus. The result was presented in Table 1.

Fermentation and metabolite extraction⁵

The fungal strain was cultured in 500 ml culture flasks containing potato dextrose broth. After 14 days of incubation at 25°C on a shaker at 160 rpm, the mycelium and broth were separated by filtration⁵ and the supernatant were centrifuged at 4000 rpm for 5 min. Mycelium free supernatant were extracted three times with equal volume of ethyl acetate. Upon solvent evaporation, ethyl acetate extract was obtained.

Isolation of bioactive compound

Preparative Thin Layer Chromatography/TLC [stationary phase = silica gel 60 PF₂₅₄; mobile phase = n-hexane: ethyl acetate: methanol (2:6:1 v/v) and 1 drop of glacial acetic acid] was applied to separate the ethyl acetate extract, resulting in four fractions that were subjected for antimicrobial test. The most active fraction⁵ was further purified using preparative TLC [stationary phase = silica gel 60 PF₂₅₄; mobile phase = chloroform: ethyl acetate (1:3 v/v)]. A TLC based pure compound was obtained, appeared as a single peak on HPLC (High Performance Liquid Chromatography).

Antimicrobial testing

Screening for anti-microbial activity was conducted against various microorganisms (Escherichia coli (ATCC 11229), Staphylococcus aureus (ATCC 25923), Bacillus subtilis (ATCC 6633), Streptococcus mutans (ATCC 25175), Pseudomonas aeruginosa (ATCC 27853) in vitro by disc diffusion method [27]. This was carried out on nutrient agar plates and conducted at least twice. The fractions obtained from preparative TLC were dissolved in ethanol, and 10 µl of a series concentration of fractions (12.5 – 200 µg/disc) were loaded into the discs. Streptomycin (100 µg/disc) and ethanol were employed to function as positive standard and negative control, respectively. Petri dishes containing medium agar were embedded with discs containing fractions as well as the controls at the surface of agar. Following incubation at 37°C for 24 h, the clear zones surrounding the discs were measured in mm and designated as the inhibition zones. The IC₅₀ and MBC (Minimum Bactericidal Concentration) of bioactive compounds were determined by modified microdilution method

[28,29]. Testing microorganisms were prepared and the densities were compared to 0.5 McFarland standard, followed by dilution (1:10) with Mueller Hinton. The microbial inoculum was transferred triplicate into 96-well plate. The sample dissolved in ethanol were serially diluted by using Mueller Hinton and transferred into microbial inoculum in 96 well plate to final concentration from 20 – 0.16 µg/ml for determining IC₅₀ and from 40 – 0.16 µg/ml for determining MBC. Controls of microbial growth, solvent and media were included in testing plate. Following overnight incubation at 37°C and the plates were read at 595 nm using microplate reader (Biorad). IC₅₀ was expressed as the concentration that inhibited 50% of growth. This was calculated from plotting percent growth and concentration of sample tested. MBC was defined as the lowest concentration that showed no growth after subculture into fresh nutrient agar.

Cytotoxic activity

MTT assay was used to assess cell viability [30]. T47D (Human ductal breast epithelial tumor cells) and Vero (Normal African green monkey kidney epithelial cells) were grown in Roswell Park Memorial Institute (RPMI) 1640. These cells were used as model for cancerous and normal cell, respectively. The media was supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin and 1% fungizone. The cultures were held in tissue culture flasks and incubated at 37°C supplied with 5% CO₂. Cells were harvested and 100 µl of cell suspensions containing 5x10³ cells were dispensed into 96-well plate. After 48 h incubation and reaching 70% – 80% confluent, various concentrations of isolated bioactive compound were added. Doxorubicin was used as positive control in this study. Following 24 h of incubation, subsequent washing with 1X Phosphate-Buffered Saline (PBS) was conducted, and 100 µl of medium containing 0.5 mg/ml 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dispensed to the well. The plates were further incubated for 4 h at 37°C followed by adding 100 µl of 10% sodium dodecyl sulfate-0.01 N HCl overnight in dark condition to dissolve the MTT formazan produced. After overnight incubation⁵, the plates were shaken, and read in microplate reader (Biorad) at 595 nm. Cells absorbance (Abs) was converted to percentage of cell viability. Percentage of cell viability = $\frac{\text{Abs of treated cells} - \text{Abs of medium} \times 100\%}{\text{Abs of control untreated cells} - \text{Abs of medium}}$. Value of IC₅₀ (concentration required for 50% inhibition) was determined from plotting percent

cell viability and concentration of isolated compound.

Selectivity index (SI) was calculated based on ratio of IC₅₀ value of compound on normal (Vero) cells versus cancer cells (T47D).³¹

Identification of chemical structure

Predicted chemical structure of bioactive compound was conducted based on analysis of UV, FT-IR, LC-MS, ¹H-NMR, ¹³C-NMR. The compound (1 mg) was diluted in methanol and subjected to UV spectrophotometer (Hitachi UH 5300) to obtain UV spectrum. Infrared spectrum was obtained by loading encapsulated compound in KBr pellet into FT-IR spectrophotometer (Perkin Elmer Spectrum 100, Perkin Elmer Life and Analytical Science, Shelton, USA). The compound was analyzed using LC-MS with an ESI source (Shimadzu, Shimadzu Corporation, Kyoto, Japan). Spectra of ¹H-NMR and ¹³C-NMR in CD₃-OD were analyzed using JEOL at 500 MHz (JEOL USA Inc., Boston, USA).

RESULTS

Antimicrobial activities

Antimicrobial assay using disc diffusion test was used as a guided assay for isolation of bioactive compound within ethyl acetate extract of endophytic fungus fermentation broth. The antimicrobial activities of four fractions obtained from preparative thin layer chromatography were conducted against *E. coli*, *S. aureus*, *B. subtilis*, *S. mutans* and *P. aeruginosa*. Fractions 1 and 3 at the given concentrations were found to be active

against two tested microorganisms (*S. aureus* and *B. subtilis*). On the other hand, other fractions (2 and 4) have broader activities against five testing microorganisms except fraction 4 which was not active against *P. aeruginosa* (Table 2). These antibacterial activities, however, were lower compared to the positive control streptomycin. Among other fractions, fraction 2 was found to be the most active reaching up to 20 mm inhibition zone against *S. subtilis* (Figure 1). The fraction 2 was further purified and tested for its IC₅₀ and MBC values.

Based on antibacterial testing, the purified fraction 2 showed IC₅₀ value of less than 2 µg/ml against all testing microorganisms (Table 3). This compound was found to be effectively killed *S. mutans*, better than streptomycin control with MBC value of 10.00 µg/ml. Higher concentration is needed to kill *S. aureus* and *P. aeruginosa* with MBC value of 40.00 µg/ml.

Cytotoxicity activities

Preliminary screening on the ability of isolated compound to inhibit the development of cancer cells was conducted by cytotoxicity testing against T47D breast cancer cells and normal Vero cells. This compound inhibited the growth of T47D (IC₅₀ value of 174 µg/ml) and Vero cells (IC₅₀ value of 214 µg/ml) with Selectivity Index 1.23; whilst the positive control doxorubicin exhibited IC₅₀ value of 4.86 ± 0.21 against T47D and 297.59 ± 10.1 µg/ml against Vero cells with Selectivity Index of 61.23.

Table 1. Identification of endophytic fungus.#

ITS_4_Reverse
TAGTCATATATGCGCCTATATACTAGATATATGCACATAGTCTTATAAGTAGTACAGATAAATTAGAATCC
CCCTTGGTATAGGCGTAGATATTATCACACCAACCGTAGGCATTTCTACATGTCCCACTAATAATTTTAA
AGAGAGCCAGTTAAGAAGTAAGTCACTCTCACATCCAAGCCTTACAAAAAATTATAAGGTTGA
GAATTTAATGACTCTCAACAGGCATGCCCCCTCGGAATACCAAAGGGCGCAAGGTGCGTTCAAAGAT
TCGATGATTCACTGGATTCTGCAATTCACATTACTTATCGCATTTGCTGCGTTCTTCATCGATGCAAGA
GCCAAGAGATCCGTTGTTGAAAGTTGATATTATTTTATTAAGAGAAACAAACCCTATATAAGAGTATGC
TTCAATATGAGATCGTTCTATGTAAACATACAATAGAGTTATATAAAGTAGTCATAGTCAGAATTTCTTCTGA
CTACAGTTGGTTCAAGGTGTATATAAGTATATAGCTCAAAGTGTGCACATGAATAAATTACCAGCA
CAACTTCTTTGCATATATATGAATCAATAATGATCCTCCGCAGGTTACCTACGGAACCTTGTTACG
ACTTTTACTCC
ITS_5_Forward
GATTCATATATATGCAAAGAAGTTGTGCTGGTAATTTATTACATGTGCACACTTTGGAGCTATATACTTAT
ATACACCTGTGAACCAACTGTAGTCAGAAGAAATTCTGACTATGACTACTTTATATAACTCTATTGTATGT
TACATAGAACGATCTCATATTGAAGCATACTTTATATAGGGTTTGTCTCTTAATGAAATAATATACAAC
TTCAACAACGGATCTCTTGGCTCTTGATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA
TTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATCCGAGGGGCGAT
GCCTGTTTGAGAGTCATTAAATTCTAACCTTATAAATTTTTTTGTAAGGCTTGGATGTGAGAGTTGCTA
GTACTTCTTAAGTCTCTTTAAATTATTAGTGGGACATGTAGAAATGCCTACGTTGGTGTGTGATA
ATATCTACGCCTATACCAAGGGGGATTCTAATTTATCTGTACTACTTATAAGACTATGTGCATATATCTAG
TATATAGGCGCATATATTGACTATTTGACCTCAAATCAGGTAGGACTACCCGCTGAACCTAAGCATATC

AAT
Contig-Sample GGAGTAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATTCATATATAT GCAAAGAAGTTGTGCTGGTAATTTATTACATGTGCACACTTTGGAGCTATATACTTATATACACCTGTGA ACCAACTGTAGTCAGAAGAAATCTGACTATGACTACTTTATATAACTCTATTGTATGTTACATAGAACGA TCTCATATTGAAGCATACTCTATATAGGGTTTGTCTCTTAATGAAATAATATACAACCTTCAACACCGG ATCTCTGGCTCTTGATCGATGAAGAACGCGAGCGAAATGCGATAAGTAATGTGAATTGCAGAATCCA GTGAATCATCGAATCTTGAACGCACCTTGCGCCCTTTGGTATTCCGAGGGGCATGCCTGTTTGAGA GTCAATAAATTCTCAACCTTATAAATTTTTTTGTAAGGCTTGGATGTGAGAGTTGCTAGTTACTTCTAACT GGCTCTCTTAAATATTAGTGGGACATGTAGAAATGCCTACGGTTGGTGTGATAATATCTACGCCTAT ACCAAGGGGGATTCTAATTTATCTGTACTACTATAAGACTATGTGCATATATCTAGTATATAGGCGCATA TATTGACTATTGACCTCAAAATCAGGTAGGACTACCCGCTGAACCTTAAGCATATCAAT
Athelia rolfsii strain orchid (*The sequence of ITS rDNA isolate and the BLAST result of the most similar taxon coded for Athelia rolfsii strain orchid., a species used as the reference. GQ358518 was the code to access in DDBJ or NCBI. [Homology: 91%; Max score: 918; Total score: 918; Query coverage: 100%; E-value: 0.0; Max identities: 632/694 (91%); Gaps: 21/694 (3%)])

Table 2. Inhibition zone of fractions against five testing microorganism using disc diffusion method.

Fractions	Loading (µg)	Inhibition zones (mm) ±SD						
		S. aureus	B. subtilis	S. mutans	E. coli	P. aeruginosa	Control (+)	Control (-)
1	12.5	ND	7.18±0.06	ND	ND	ND	21.68±0.76	ND
	25	7.55±0.1	9.07±0.18	ND	ND	ND		
	50	1	16.46±0.5	ND	7.96±0.57	ND		
	100	ND	6	ND	ND	ND		
	200	11.52±0.22	7.12±0.13	ND	ND	18.83±0.89		
2	12.5	8.65±0.1	20.86±1.0	5.41±0.33	14.16±0.65	7.72±0.38	22.54±0.64	ND
	25	0	3	12.65±0.6	16.21±0.52	10.68±0.4		
	50	15.01±0.	18.92±0.9	2	16.84±0.16	2		
	100	10	5	15.70±0.4	16.71±0.23	11.60±0.4		
	200	15.48±0.28	17.53±0.3	3	17.89±0.10	5		
3	12.5	ND	ND	ND	ND	ND	22.32±1.91	ND
	25	ND	ND	ND	ND	ND		
	50	12.83±0.	19.76±0.6	ND	ND	ND		
	100	64	8	ND	ND	ND		
	200	8.75±0.4	18.25±0.5	ND	18.9±0.72	ND		
4	12.5	ND	ND	11.36±0.4	ND	ND	21.25±0.82	ND
	25	ND	7.55±0.43	5	10.15±0.59	ND		
	50	13.12±0.	12.22±1.0	10.42±0.5	12.69±0.70	ND		
	100	96	7	1	15.22±1.13	ND		
	200	13.87±0.11	12.14±1.1	10.69±1.0	16.34±0.45	ND		
		9.09±0.3	9.88±0.97	11.19±0.6				

		6		5				
				10.66±0.6				
				5				

ND = not detected

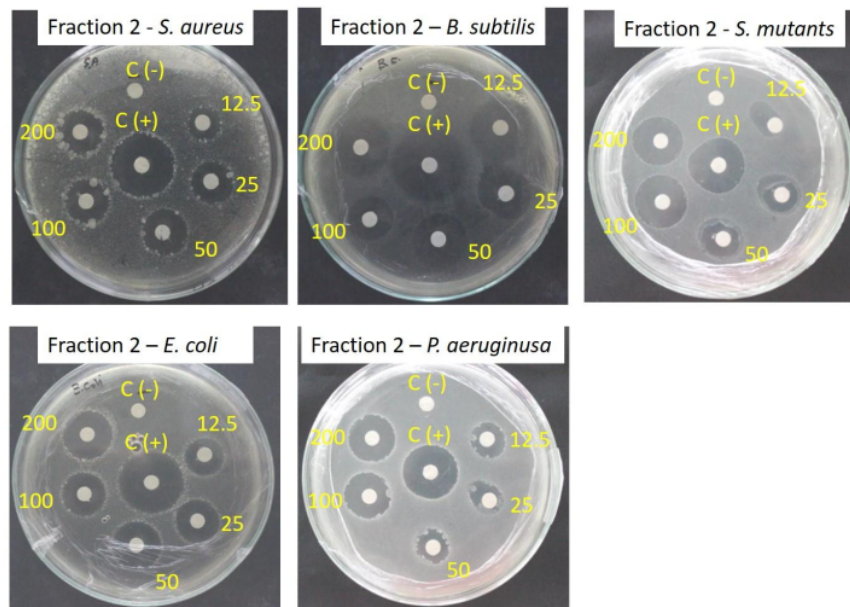


Fig.1:Antibacterial activity of fraction 2 as determined by disc diffusion test.
C(-) = negative control. C (+) = positive control, streptomycin. The samples were added at µg/disc.

Table 3. IC₅₀ and MBC values of isolated compound

Microorganisms	Compound	
	IC ₅₀ ±SD(µg/ml)	MBC (µg/ml)
S. aureus	0.73±0.04	40.00±0.00
B. subtilis	1.24±0.10	20.00±2.35
S. mutans	0.31±0.06	10.00±0.00
E. coli	1.17±0.03	20.00±2.35
P. aeruginosa	1.27±0.12	40.00±1.50
Streptomycin	0.81±0.02	20.00±2.25

Characteristics of isolated compound

Purification of fraction 2 using preparative thin layer chromatography resulted in white crystalline powder having melting point of 175.21 – 175.90^o C. Liquid chromatography analysis of this compound exhibited 100% level of purity with retention time of 2.2 minutes (Figure 2). Ultraviolet spectrum (MeOH) showed λ maximum absorption at 289.5 nm indicating the present of an aromatic moiety (Figure 2). Infrared spectrum (KBr, cm⁻¹) of the compound (Figure 2) showed the present of a broad absorption band at 3500-3200 cm⁻¹ indicating the present of –OH group, and at 1650 cm⁻¹ indicating the present of an α, β unsaturated C=O group. The ¹³C-NMR spectrum

of isolated compound (δ, ppm, CD₃OD) (Table 4; Figure 3) displayed signals of an –C=O ester (δ, 177.68 ppm) and 6 aromatic carbon signals (δ, 140.11, 137.34, 132.56, 129.34, 115.41, 110.36) in which one (δ, 129.87) of those was a substituted aromatic carbon. Two unsaturated carbons signals appeared at δ, 110.36 and 100.10 ppm, a –OCH₃ signal appeared at δ, 70.12 ppm, a downfield signal of a –CH at δ, 57.76 ppm and 2 –CH signal (δ, 37.10; 30.56 ppm) and a methyl carbon signal at δ 22.10 ppm.

The ¹H-NMR (δ, ppm, CD₃OD) of isolated compound demonstrated the present of aromatic proton signals [δ, 7.85 ppm and 7.84, dd (7.75

Hz)] substituted by heteroatom (Table 4, Figure 4). A characteristic singlet signal at δ , 3.67 supposed to be a $-\text{OCH}_3$ signal. There were plenty of multiplet signals at the more upfield region that were assigned as methine and methyl signals (δ , 2.00 – 0.80 ppm). Proton signals at δ 1.20, 1.21, and 1.22, triplet with integration ratio 1 were suggested to be methine ($-\text{CH}-$) signal. Proton signals at δ 2.16, 2.17, 2.19, 2.21 and 2.24 ppm, quintet (5.05 Hz) with integration ratio 1 were also suggested to be ($-\text{CH}-$). The presence of methylene groups were detected on

various proton signals [δ , 2.0 ppm, d(5.10 Hz); δ , 1.62 ppm, s; δ , 1.29, 1.30, 1.32, 1.39 ppm, quartet (5.05 Hz); δ , 1.11, 1.12, 1.13, 1.142, 1.149, 1.15 ppm, sextet (5 Hz)]. Proton signals at δ 0.88, 0.90 and 0.91, t (5 Hz) were supposed to be methyl ($-\text{CH}_3$).

The mass spectrum (ESI LCMS) (Figure 4) displayed a base peak at m/z 221.2967. Based on combination data (IR, MS, ^{13}C - and ^1H - NMR), chemical structure of isolated compound was suggested to be an aromatic compound having methoxy, hydroxyl and methyl group.

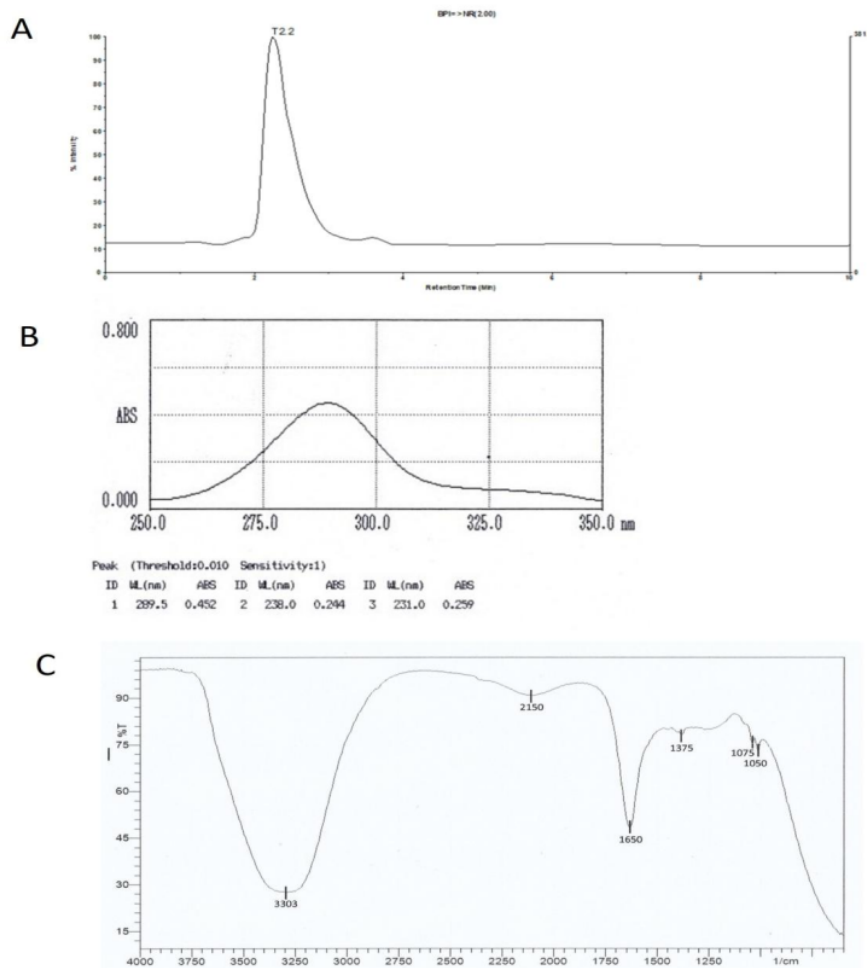


Fig.2:Liquid Chromatography profile of isolated compound. System: reverse phase. Mobile phase: methanol: water (90:10 v/v). Stationary phase: c18. Flow rate: 1 ml/minute (A); UV (MeOH) (B); and FT-IR (KBr) (C) spectra.

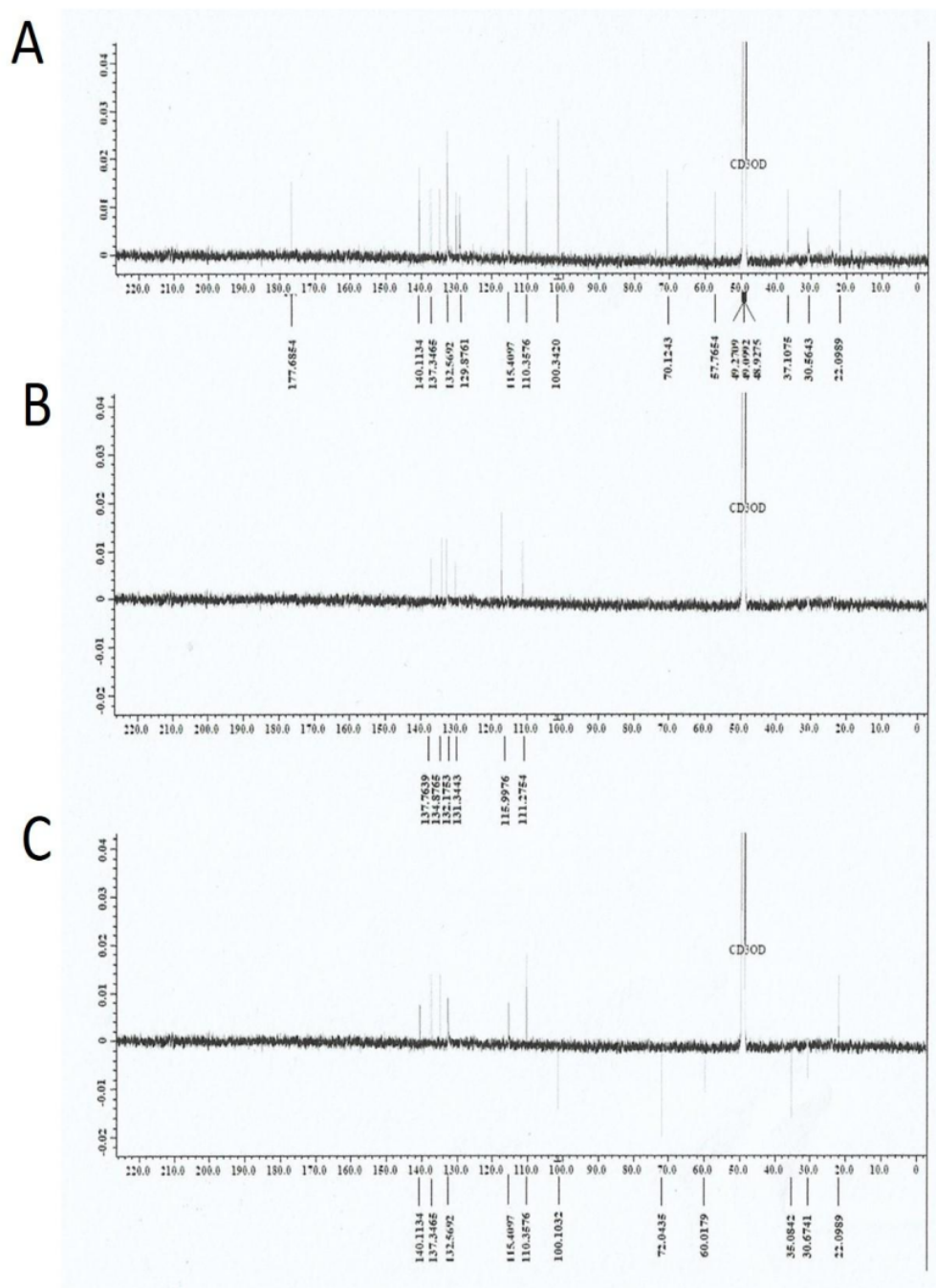


Fig.3: ¹³C-NMR (CD₃OD, 500 MHz) (A), DEPT-90 (B), DEPT-135 (C) of isolated compound

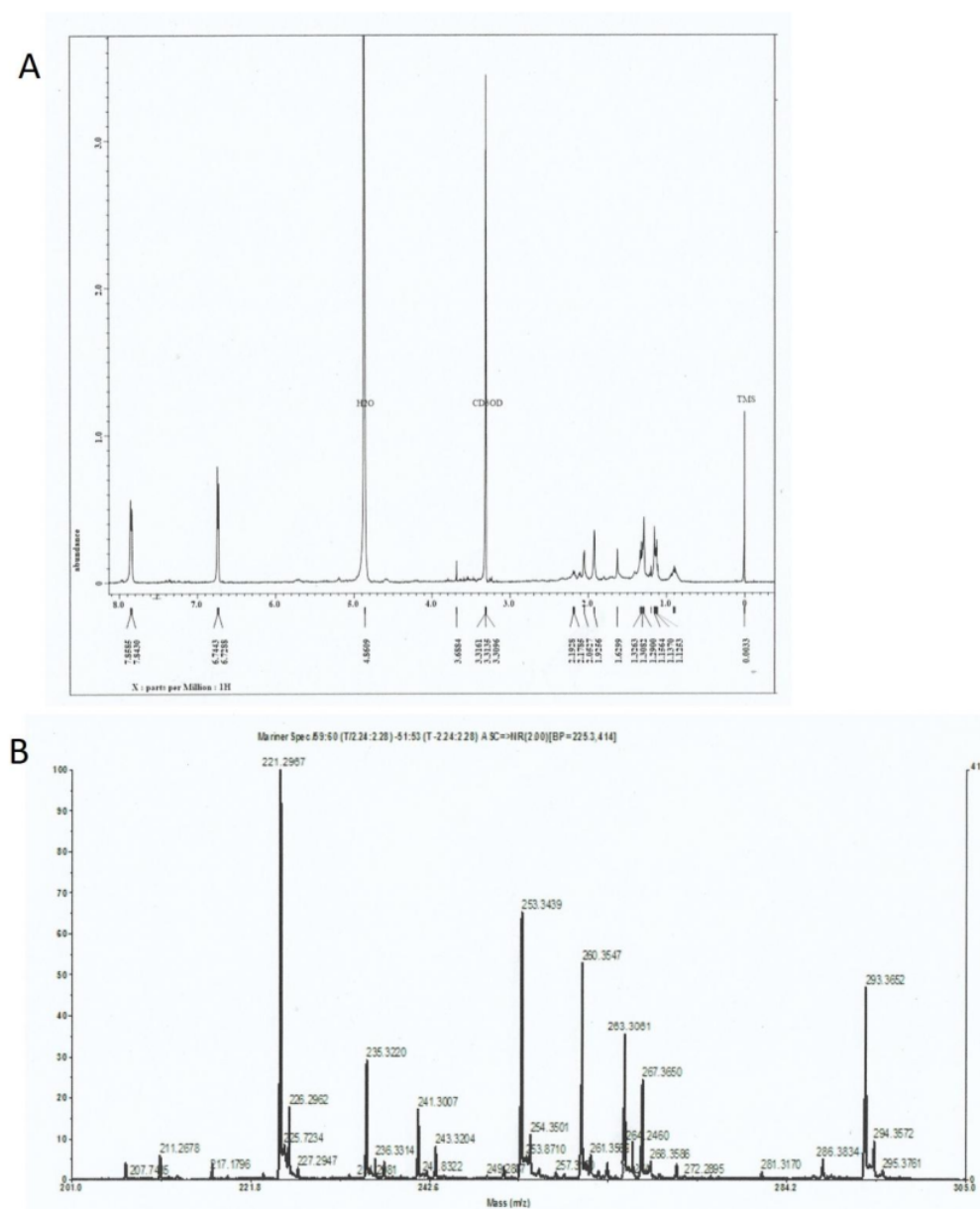


Fig.4: ¹H-NMR of isolated compound (CD₃OD, 500 MHz) (A); Mass spectrum of isolated compound (ESI-LCMS) (B)

Table 4. The ¹H and ¹³C NMR spectral data of isolated compound.

¹ H NMR (δ ppm)	¹³ C NMR (δ ppm)	Predicted functional groups
3.68 (singlet)	177.68 70.12	-C=O ester -OCH ₃
2.16, 2.17, 2.19, 2.21 and 2.24 (quintet, J = 5.05 Hz)	57.76	-CH-

1.20, 1.21, and 1.22 (triplet)	37.10	-CH=
2.05 (d, J = 5.10 Hz)	30.67, 35.08,	-CH2-
1.62 (singlet)	60.02, 72.04,	
1.29, 1.30, 1.32, 1.39 (quartet J = 5.05 Hz)	100.10	
1.11, 1.12, 1.13, 1.142, 1.149, 1.15 (sextet, J = 5 Hz)		
	110.36;100.10	-C=C-
7.85 and 7.84, (dd, J = 7.75 Hz)	140.11, 137.34, 132.56, 129.34, 115.41, 110.36)	Aromatic carbon substituted heteroatom
0.88; 0.90; 0.91 (triplet, J = 5 Hz)	22.10	-CH3

DISCUSSION

The need to search lead compounds having antimicrobial values comes from the evolutionary response of microbes towards existing antibiotics with subsequent increased rates of resistance [32]. Similarly, the need to find novel and effective therapeutic compounds against cancer with minimum side effects is of scientific challenge [33]. Previous study found potential endophytic fungi isolated from *C. amboinicus* producing antimicrobials [34] with one of them identified as *Athelia rolfsii* (Table 1). Further characterization was conducted by screening bioactive compounds within ethyl acetate fractions of the culture medium separated by preparative thin layer chromatography against five testing microorganisms. The testing microorganisms used in this study representing standard gram positive and negative bacteria.

Bioassay guided isolation using disc diffusion test showed that fraction 2 was found to be active against all testing microorganisms. The inhibition zones, however, were not dose dependent suggested that at a higher concentration some fractions might not diffuse well within the solid medium. This could be contributed by the solubility or polarity of compounds within the fractions in which non-polar compounds did not diffuse well in water medium [35]. Further isolation and characterization of the bioactive compound within fraction 2 showed that in general, the isolated compound mostly, if not all, was more active against gram positive bacteria with the best IC_{50} value against *S. mutant*, a commonly recognized pathogen of dental caries, followed by *S. aureus*. The IC_{50} values exceeded the IC_{50} values of streptomycin control. The ability of the isolated compound in inhibiting the growth of bacteria were even better than those reportedly isolated from *N. nouchali* endophytic fungi and were comparable to that (plumbagin) isolated from endophytic fungi *Cladosporium delicatulum* [36,37].

Structure elucidation of the isolated compound appeared as an aromatic compound having methoxy, hydroxyl and methyl groups. The presence of phenolic -OH group in its chemical structure may contribute in the antibacterial activities [38,39]. Based on its IC_{50} values which were less than 2 μ g/ml in all tested microorganisms indicated that the isolated compound was potential for further development as anti-infective agents. Its chemical structure elucidation, however, need also to be confirmed by 2D NMR data.

Considering its bioactivity against microbes, in this study, preliminary screening on the ability of the isolated compound to inhibit the growth of cancer cells also was conducted. Some antibiotics were reported to have ability to kill cancer cells. Salinomycin for example, an antibiotic extracted from *Streptomyces albus* was found to be able to kill PC-3 cells and its cancer stem cell derivatives in vitro and in vivo [40]. This compound had shown to prevent the development of many cancer cells including those which are multi-drug resistance as well as cancer stem cells [41]. Similarly, Actinomycin D, a well-known antibiotic of the actinomycin group, had been reportedly to be used for treatment of sarcomas, and trophoblastic tumors [42,43] and had been found to induce apoptosis of small cell lung cancer, non-small cell lung cancer, pancreatic cancer cell and breast cancer stem cell [44,46].

This study found that the isolated compound prevented the growth of T47D breast cancer cell line showing IC_{50} of 174 μ g/ml and exhibited higher IC_{50} value when it was tested against normal Vero cells. Selectivity index of less than 3, however, suggesting that this compound was not selective [31]. Based on the screening endpoints for cytotoxic activities used by Mahavorasirikul et al., pure compounds were considered potential cytotoxic if the IC_{50} value is less than 4 μ g/ml [47]. This study demonstrated the weak cytotoxic

activity of the isolate against T47D cells. This might be explained by the presence of gamma-delta unsaturated carbonyl instead of alpha-beta unsaturated carbonyl. The contribution of alpha, beta unsaturated carbonyl moiety to the cytotoxic activities of various simple alpha, beta unsaturated carbonyl compounds had been reported against cultured human normal and oral tumor cells [48]. Further study against other panel of cancer cell lines was needed to confirm this finding.

CONCLUSION

A bioactive compound had been obtained from the fermentation broth of an endophytic fungus of *C. amboinicus* and it displayed promising antimicrobial activity. This compound, however, showed weak cytotoxicity towards T47D cells and was not selective against normal cell; therefore, it may be potential to be developed as lead compound for antimicrobial agent.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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REFERENCES

1. Deshmukh SK, Gupta MK, Prakash V, Reddy MS, Mangrove-Associated Fungi: A Novel Source of Potential Anticancer Compounds, *J Fungi (Basel)*, 2018;4(3):pii:E101.
2. Gill H, Vasundhara M, Isolation of taxol producing endophytic fungus *Alternaria brassicicola* from non-Taxus medicinal plant *Terminalia arjuna*, *World J Microbiol Biotechnol*, 2019;35(5):74.
3. Goutam J, Sharma G, Tiwari VK, Mishra A, Kharwar RN, Ramaraj V et al., Isolation and Characterization of "Terrein" an Antimicrobial and Antitumor Compound from Endophytic Fungus *Aspergillus terreus* (JAS-2) Associated from *Achyranthus aspera* Varanasi, India, *Front Microbiol*, 2017;8:1334.
4. Shiono Y, Muslihah NI, Suzuki T, Ariefa NR, Anwar C, Nurjanto HH et al., New eremophilane and dichlororesorcinol derivatives produced by endophytes isolated from *Ficus ampelas*, *J Antibiot (Tokyo)*, 2017;70(12):1133-1137.
5. Zhang H, Wang X, Li R, Sun X, Sun S, Li Q et al., Preparation and Bioactivity of Exopolysaccharide from an Endophytic Fungus *Chaetomium* sp. of the Medicinal Plant *Gynostemma pentaphylla*, *Pharmacogn Mag*, 2017;13(51):477-482.
6. Zhou I, Diao X, Wang T, Chen G, Lin Q, Yang X et al., Phylogenetic diversity and antioxidant activities of culturable fungal endophytes associated with the mangrove species *Rhizophora stylosa* and *R. mucronata* in the South China Sea, *PLoS One*, 2018;13(6):e0197359.
7. Stierle A, Strobel G, Stierle D, Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew, *Science*, 1993;260(5105):214-216.
8. Kusari S, Zühlke S, Spiteller M, An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues, *J Nat Prod*, 2009;72:2-7.
9. Mousa WK, Raizada MN, The diversity of antimicrobial secondary metabolites produced by fungal endophytes: an interdisciplinary perspective, *Front Microbiol*, 2013;4:65.
10. Sebastianes FL, Cabedo N, El Aouad N, Valente AM, Lacava PT, Azevedo JL et al., 3-hydroxypropionic acid as an antibacterial agent from endophytic fungi *Diaporthe phaseolorum*, *Curr Microbiol*, 2012;65(5):622-632.
11. Verma VC, Lobkovsky E, Gange AC, Singh SK, Prakash S, Piperine production by endophytic fungus *Periconia* sp. isolated from *Piper longum* L, *J Antibiot (Tokyo)*, 2011;64(6):427-431.
12. Pinheiro EA, Carvalho JM, dos Santos DC, Feitosa Ade O, Marinho PS, Guilhon GM et al., Antibacterial activity of alkaloids produced by endophytic fungus *Aspergillus* sp. EJC08 isolated from medical plant *Bauhinia guianensis*, *Nat Prod Res*, 2013;27(18):1633-1638.
13. Chutulo EC, Chalannavar RK, Endophytic Mycoflora and Their Bioactive Compounds from *Azadirachta indica*: A Comprehensive Review, *J Fungi (Basel)*, 2018; 4(2):pii, E42.
14. Wu H, Yang H, You X, Li Y, Isolation and Characterization of Saponin-Producing Fungal Endophytes from *Aralia elata* in Northeast China, *Int J Mol Sci*, 2012; 13(12):16255-16266.
15. Hullatti K, Bhattacharjee P, Pharmacognostical Evaluation of Different Parts of *Coleus amboinicus* Lour., *Lamiaceae*, *Pharmacogn J*, 2011;3:39-44.
16. Girish K, Antimicrobial activities of *Coleus aromaticus* Benth, *J Pharm Res*, 2016;10(10):635-646.

17. Arumugam G, Swamy MK, Sinniah UR, Plectranthus amboinicus (Lour.) Spreng: Botanical, Phytochemical, Pharmacological and Nutritional Significance, Molecules, 2016;21(4):369.
18. Vasconcelos ECB, Melo HM, Cavalcante TTA, Júnior FEAC, de Carvalho MG, Menezes FGR et al., Plectranthus amboinicus essential oil and carvacrol bioactive against planktonic and biofilm of oxacillin- and vancomycin-resistant Staphylococcus aureus, BMC Complement Altern Med, 2017;17:462.
19. Hasibuan PAZ, Sumaiyah S, The Anti-Proliferative and Pro-Apoptotic Properties of Ethanol Plectranthus amboinicus (Lour.) Spreng. Leaves Ethanol Extract Nanoparticles on T47D Cell Lines, Asian Pac J Cancer Prev, 2019;20(3):897-901.
20. Eduardo M. Brandão EM, Brandão PHDM, Souza IA, Paiva GS, Carvalho MC et al., Antineoplastic Effect of Aqueous Extract of Plectranthus Amboinicus in Ehrlich Ascites Carcinoma, J Cancer, 2013;4(7):573-576.
21. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J Clin, 2018;68(6):394-424.
22. Akova M, Epidemiology of antimicrobial resistance in bloodstream infections, Virulence, 2016;7(3):252-266.
23. Ding T, Jiang T, Zhou J, Xu L, Gao ZM, Evaluation of antimicrobial activity of endophytic fungi from Camptotheca acuminata (Nyssaceae), Genet Mol Res, 2010;9(4):2104-12.
24. O'Donnell K, Fusarium and its near relatives. In: Reynolds DR and Taylor JW, eds. The fungal holomorph: Mitotic, meiotic, and pleomorphic specification in fungal systematics. Wallingford: CAB International; 1993.
25. White TJ, Bruns TD, Lee SB, Taylor JW, Amplification and direct sequencing of fungal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols. San Diego: Academic; 1990.
26. Hiraishi A, Kamagata, Y, Nakamura, N, Polymerase chain reaction amplification and restriction fragment length polymorphism analysis of 16S rRNA genes from methanogens, J Ferment Bioengineer, 1995;79:523-529.
27. Bauer AW, Kirby WM, Sherris JC, Turck M, Antibiotic susceptibility testing by a standardized single disk method, Tech Bull Regist Med Technol, 1966; 36(3):49-52.
28. da Silva Filho AA, de Sousa JP, Soares S, Furtado NA, Andrade e Silva ML, Cunha WVR et al., Antimicrobial activity of the extract and isolated compounds from Baccharis dracunculifolia D. C. (Asteraceae), Z Naturforsch C, 2008;63(1-2):40-46.
29. Muhammad I, Li XC, Jacob MR, Tekwani BL, Dunbar DC, Ferreira D, Antimicrobial and antiparasitic (+)-trans-hexahydrodibenzopyrans and analogues from Machaerium multiflorum, J Nat Prod, 2003;66:804D809.
30. Nugroho AE, Hermawan A, Putri DD, Novika A, Meiyanto E, Kawaichi M. Combinational effects of hexane insoluble fraction of Ficus septica Burm. F. and doxorubicin chemotherapy on T47D breast cancer cells, Asian Pac J Trop Biomed, 2013; 3(4):297-302.
31. Prayong P, Barusru S, Weerapreeyakul N, Cytotoxic activity screening of some indigenous Thai plants, Fitoterapia, 2008;79(7-8):598-601.
32. Sykes R, The 2009 Garrod Lecture: the evolution of antimicrobial resistance: a Darwinian perspective, J Antimicrob Chemother, 2010;65:1842-1852.
33. Siegel RD, Naishadham D, Jemal A, Cancer statistics, CA Cancer J Clin, 2012;62:10-29.
34. Astuti P, Sudarsono, Nisak K, Nugroho GW, Endophytic Fungi Isolated from Coleus amboinicus Lour Exhibited Antimicrobial Activity, Adv Pharm Bull, 2014;4(Suppl 2):599-605.
35. Cos P, Vlietinck AJ, Berghe DV, Maes L, Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept', J Ethnopharmacol, 2006;106:290-302.
36. Dissanayake RK, Ratnaweera PB, Williams DE, Wijayarathne CD, Wijesundera RLC, Andersen RJ et al., Antimicrobial activities of endophytic fungi of the Sri Lankan aquatic plant Nymphaea nouchali and chaetoglobosin A and C, produced by the endophytic fungus Chaetomium globosum, Mycology, 2016;7(1):1-8.
37. Venkateswarulu N, Shameer S, Bramhachari PV, Thaslim Basha SK, Nagaraju C, Vijaya T, Isolation and characterization of plumbagin (5-hydroxyl-2-methylnaphtalene-1,4-dione) producing endophytic fungi Cladosporium delicatulum from endemic medicinal plants, Biotechnol Rep (Amst), 2018;20:e00282.
38. Kim MK, Park JC, Chong Y, Aromatic hydroxyl group plays a critical role in antibacterial activity of the curcumin analogues, Nat Prod Commun, 2012;7(1):57-58.
39. Ultee A, Bennik MHJ, Moezelaar R, The Phenolic Hydroxyl Group of Carvacrol Is Essential for Action against the Food-Borne Pathogen Bacillus cereus, Appl Environ Microbiol, 2002; 68(4):1561-1568.
40. Zhang Y, Liu L, Li F, Wu T, Jiang H, Jiang X et al., Salinomycin Exerts Anticancer Effects on PC-3 Cells and PC-3-Derived Cancer Stem Cells In Vitro and In Vivo, Biomed Res Int, 2017;4101653.

41. Antoszczak M, Huczyński A, Anticancer Activity of Polyether Ionophore-Salinomycin, *Anticancer Agents Med Chem*, 2015;15(5):575-591.
42. Estlin EJ, Veal GJ, Clinical and cellular pharmacology in relation to solid tumours of childhood, *Cancer Treat Rev*, 2003;29:253-273.
43. Tattersall MH, Sodergren JE, Dengupta SK, Trites DH, Modest EJ, Frei E. 3rd. Pharmacokinetics of actinomycin D in patients with malignant melanoma, *Clin Pharmacol Ther*, 1975;17:701-708.
44. Das T, Nair RR, Green R, Padhee S, Howell M, Banerjee J et al., Actinomycin D Down-regulates SOX2 Expression and Induces Death in Breast Cancer Stem Cells, *Anticancer Res*, 2017;37(4):1655-1663.
45. Olberding KE, Wang X, Zhu Y, Pan J, Rai SN, Li C, Actinomycin D synergistically enhances the efficacy of the BH3 mimetic ABT-737 by downregulating Mcl-1 expression, *Cancer Biol Ther*, 2010;10:918-929.
46. Xu H, Krystal GW, Actinomycin D decreases Mcl-1 expression and acts synergistically with ABT-737 against small cell lung cancer cell lines, *Clin Cancer Res*, 2010;16:4392-4400.
47. Mahavorasirikul W, Viyanant V, Chaijaroenkul W, Itharat A, Na-Bangchang K, Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells in vitro, *BMC Complement Altern Med*, 2010;10:55.
48. Nakayachi T, Yasumoto E, Nakano K, Morshed SR, Hashimoto K, Kikuchi H et al., Structure-Activity Relationships of α , β -unsaturated Ketones as Assessed by their Cytotoxicity against Oral Tumor Cells, *Anticancer Res*, 2004;24:737-742.

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