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Research Article

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 µg/ml against S. aureus, B. subtilis, E. coli, S. mutans, and P. aeruginosa respectively. This compound also killed T47D (IC_{50} of 174 µg/ml) and Vero cells (IC_{50} of 214 µg/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].

Coleus amboinicus Lour is a medicinal plant used traditionally to treat various diseases such as urinary tract infection, digestive disorder, malaria, cough, astma 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]. Despites its medicinal uses as antimicrobial agents, C. amboinicus also had potential as anti-tumor. 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 F_{254} (Merck, Darmstadt, Germany), Silica gel 60 PF_{254} 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 an 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 extraction

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 filtration 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 PF_{254}; 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 fraction was further purified using preparative TLC [stationary phase = silica gel 60 PF_{254}; 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 37^0 C for 24 h, the clear zones surrounding the discs were measures in mm and designated as the inhibition zones. The IC_{50} 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 IC_{50} 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). IC_{50} 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% CO_2. Cells were harvested and 100 µl of cell suspensions containing $5x10^3$ 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-di-phenyltetrazolium bromide (MTT) was dispensed to the well. The plates were further incubated for 4 h at 37°C followed by adding 100 µl of10% sodium dodecyl sulfate-0.01 N HCl overnight in dark condition to dissolve the MTT formazan produced. After overnight incubation, 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 = Abs of treated cells– Abs of mediumX 100%
Abs of control untreated cells – Abs of medium

Value of IC_{50} (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 IC_{50} 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, ^{1}H-NMR, ^{13}C-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 IC_{50} and MBC values.

Based on antibacterial testing, the purified fraction 2 showed IC_{50} 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 (IC_{50} value of 174 μg/ml) and Vero cells (IC_{50} value of 214 μg/ml) with Selectivity Index 1.23; whilst the positive control doxorubicin exhibited IC_{50} 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 AGAGAGCCAGTTAAGAAGTAACTAGCAACTCTCACATCCAAGCCTTACAAAAAAAATTTATAAGGTTGA GAATTTAATGACTCTCAAACAGGCATGCCCCTCGGAATACCAAAGGGCGCAAGGTGCGTTCAAAGAT TCGATGATTCACTGGATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCAAGA GCCAAGAGATCCGTTGTTGAAAGTTGTATATTATTTCATTAAGAGAAACAAACCCTATATAAGAGTATGC TTCAATATGAGATCGTTCTATGTAACATACAATAGAGTTATATAAAGTAGTCATAGTCAGAATTTCTTCTGA CTACAGTTGGTTCACAGGTGTATATAAGTATATAGCTCCAAAGTGTGCACATGTAATAAATTACCAGCA CAACTTCTTTGCATATATATGAATTCAATAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACG ACTTTTACTCC
ITS_5_Forward GATTCATATATATGCAAAGAAGTTGTGCTGGTAATTTATTACATGTGCACACTTTGGAGCTATATACTTAT ATACACCTGTGAACCAACTGTAGTCAGAAGAAATTCTGACTATGACTACTTTATATAACTCTATTGTATGT TACATAGAACGATCTCATATTGAAGCATACTCTTATATAGGGTTTGTTTCTCTTAATGAAATAATATACAAC TTTCAACAACGGATCTCTTGGCTCTTGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA TTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATTCCGAGGGGCAT GCCTGTTTGAGAGTCATTAAATTCTCAACCTTATAAATTTTTTTTGTAAGGCTTGGATGTGAGAGTTGCTA GTTACTTCTTAACTGGCTCTCTTTAAAATTATTAGTGGGACATGTAGAAATGCCTACGGTTGGTGTGATA ATATCTACGCCTATACCAAGGGGGATTCTAATTTATCTGTACTACTTATAAGACTATGTGCATATATCTAG TATATAGGCGCATATATTGACTATTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATC

AAT
Contig-Sample GGAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATTCATATATAT GCAAAGAAGTTGTGCTGGTAATTTATTACATGTGCACACTTTGGAGCTATATACTTATATACACCTGTGA ACCAACTGTAGTCAGAAGAAATTCTGACTATGACTACTTTATATAACTCTATTGTATGTTACATAGAACGA TCTCATATTGAAGCATACTCTTATATAGGGTTTGTTTCTCTTAATGAAATAATATACAACTTTCAACAACGG ATCTCTTGGCTCTTGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATCCA GTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATTCCGAGGGGCATGCCTGTTTGAGA GTCATTAAATTCTCAACCTTATAAATTTTTTTTGTAAGGCTTGGATGTGAGAGTTGCTAGTTACTTCTTAACT GGCTCTCTTTAAAATTATTAGTGGGACATGTAGAAATGCCTACGGTTGGTGTGATAATATCTACGCCTAT ACCAAGGGGGATTCTAATTTATCTGTACTACTTATAAGACTATGTGCATATATCTAGTATATAGGCGCATA TATTGACTATTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAAT
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 spesies 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 25 50 100 200	ND 7.55±0.11 ND 11.52±0.22 12±0.66	7.18±0.06 9.07±0.18 16.46±0.56 7,12±0.13 16.28±0.51	ND ND ND ND ND	ND ND 7.96±0.57 ND ND	ND ND ND ND 18.83±0.89	21.68±0.76	ND
2	12.5 25 50 100 200	8.65±0.10 15.01±0.10 15.48±0.28 15.50±0.62 15.79±0.38	20.86±1.03 18.92±0.95 17.53±0.31 16.14±1.47 17.62±0.44	5.41±0.33 12.65±0.62 15.70±0.43 17.55±0.33 18.70±0.44	14.16±0.65 16.21±0.52 16.84±0.16 16.71±0.23 17.89±0.10	7.72±0.38 10.68±0.42 11.60±0.45 16.40±0.44 17.65±0.40	22.54±0.64	ND
3	12.5 25 50 100 200	ND ND 12.83±0.64 8.75±0.48 9.65±0.45	ND ND 19.76±0.68 18.25±0.55 9.59±0.68	ND ND ND ND ND	ND ND ND ND 18.9±0.72	ND ND ND ND ND	22.32±1.91	ND
4	12.5 25 50 100 200	ND ND 13.12±0.96 13.87±0.11 9.09±0.3	ND 7.55±0.43 12.22±1.07 12.14±1.16 9.88±0.97	11.36±0.45 10.42±0.51 10.69±1.01 11.19±0.6	ND 10.15±0.59 12.69±0.70 15.22±1.13 16.34±0.45	ND ND ND ND ND	21.25±0.82	ND

		6		5 10.66±0.65				

ND = not detected

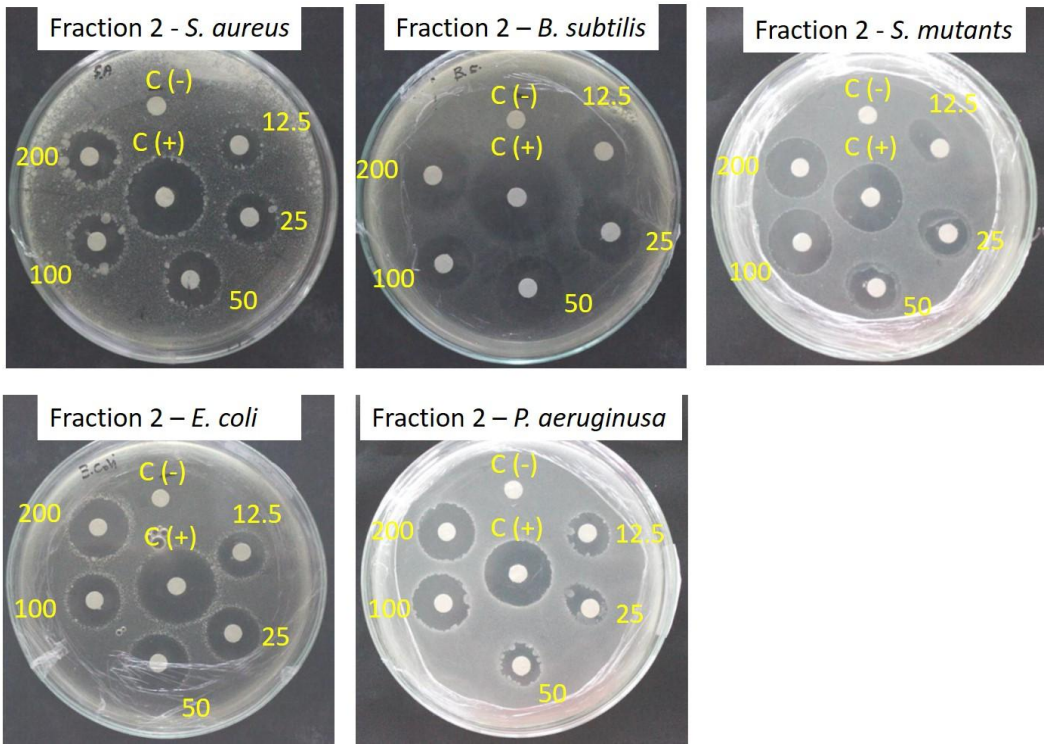

Fig.1:Antibacterial activity of fraction 2 as determined by disc diffusion test. C(-) = negative control. C (+) = positive control, streptomycin. The samples were added at µg/disc.

Table 3. IC_{50} 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 criystalline powder having melting point of 175.21 – 175.90º 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 ƛ 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 ^{13}C-NMR spectrum of isolated compound (δ, ppm, CD_3OD) (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 -OCH_3 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 ^{1}H-NMR (δ, ppm, CD_3OD) 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 $–OCH_3$ 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 (-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 (-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 (-CH3).

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 ^{1}H- NMR), chemical structure of isolated compound was suggested to be an aromatic compound having methoxy, hydroxyl and methyl group.

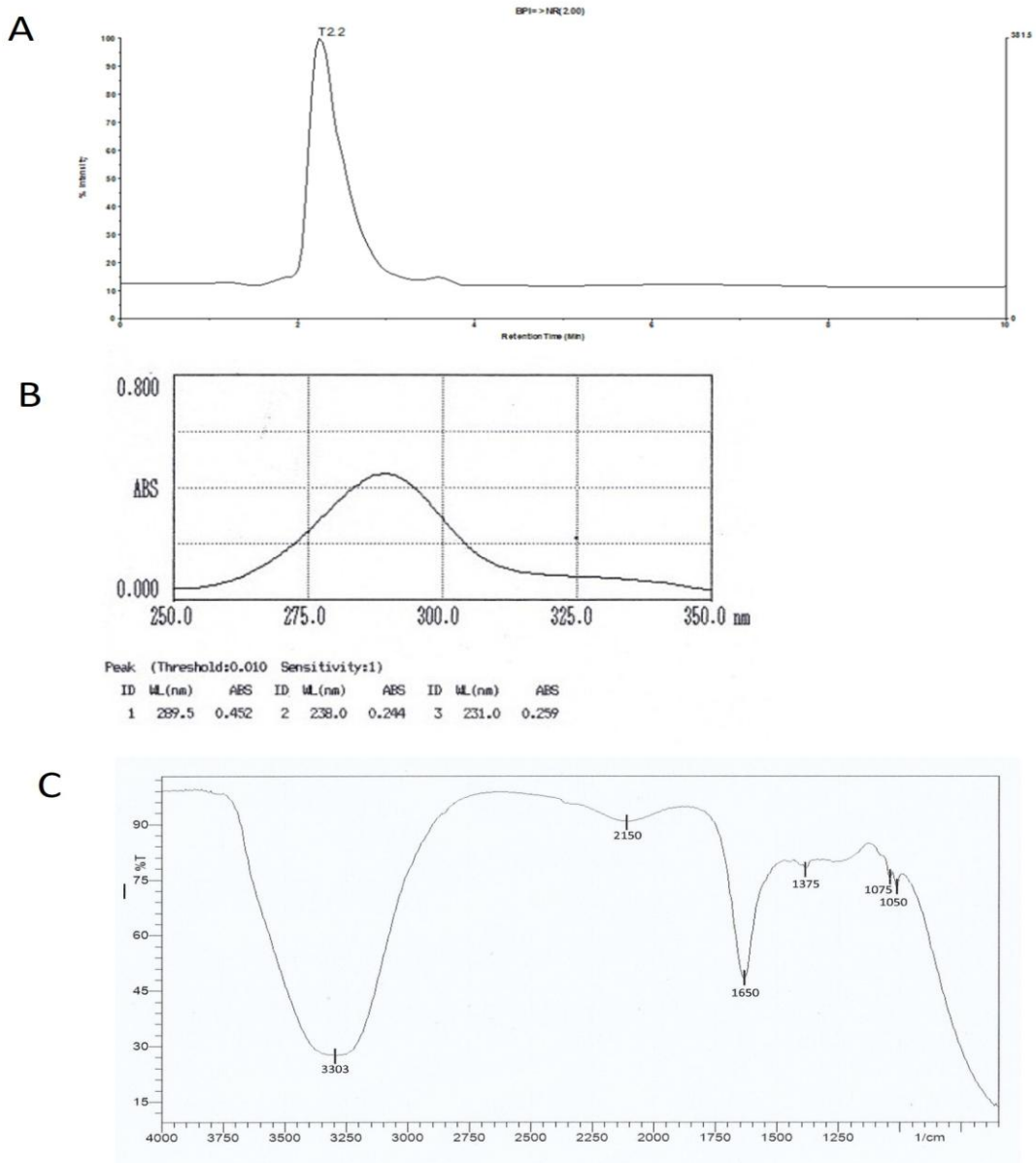

Fig.2:Liquid Chromatography profile of isolated compound. System: reverse phase. Mobile phase: methanol: water (90:10 v/v). Stationary phase: c18. Flow rate: 1 ml/minute (A); UV (MeOH) (B); and FT-IR (KBr) (C) spectrums.

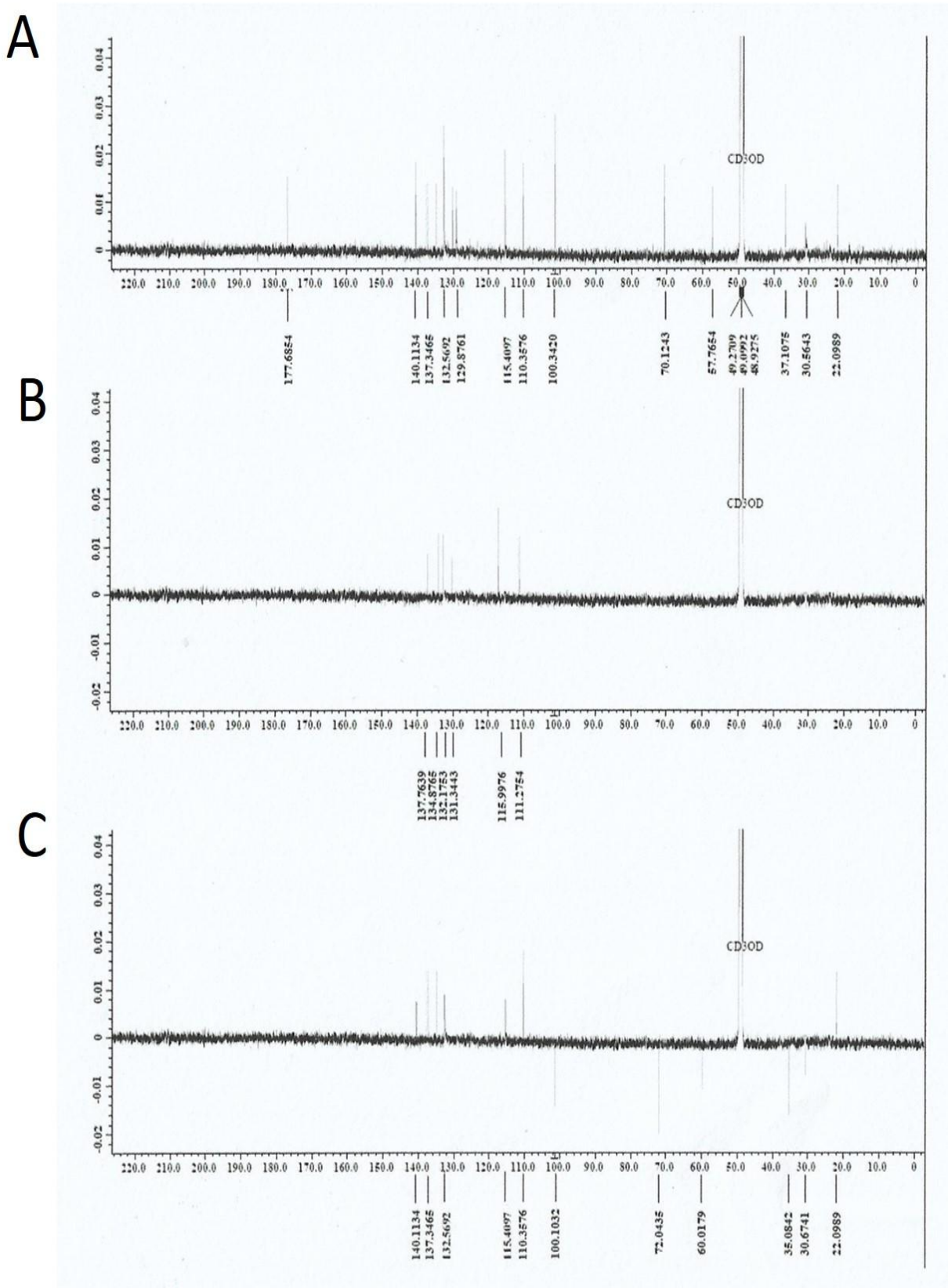

Fig.3: ^{13}C-NMR (CD_3OD, 500 MHz) (A), DEPT-90 (B), DEPT-135 (C) of isolated compound

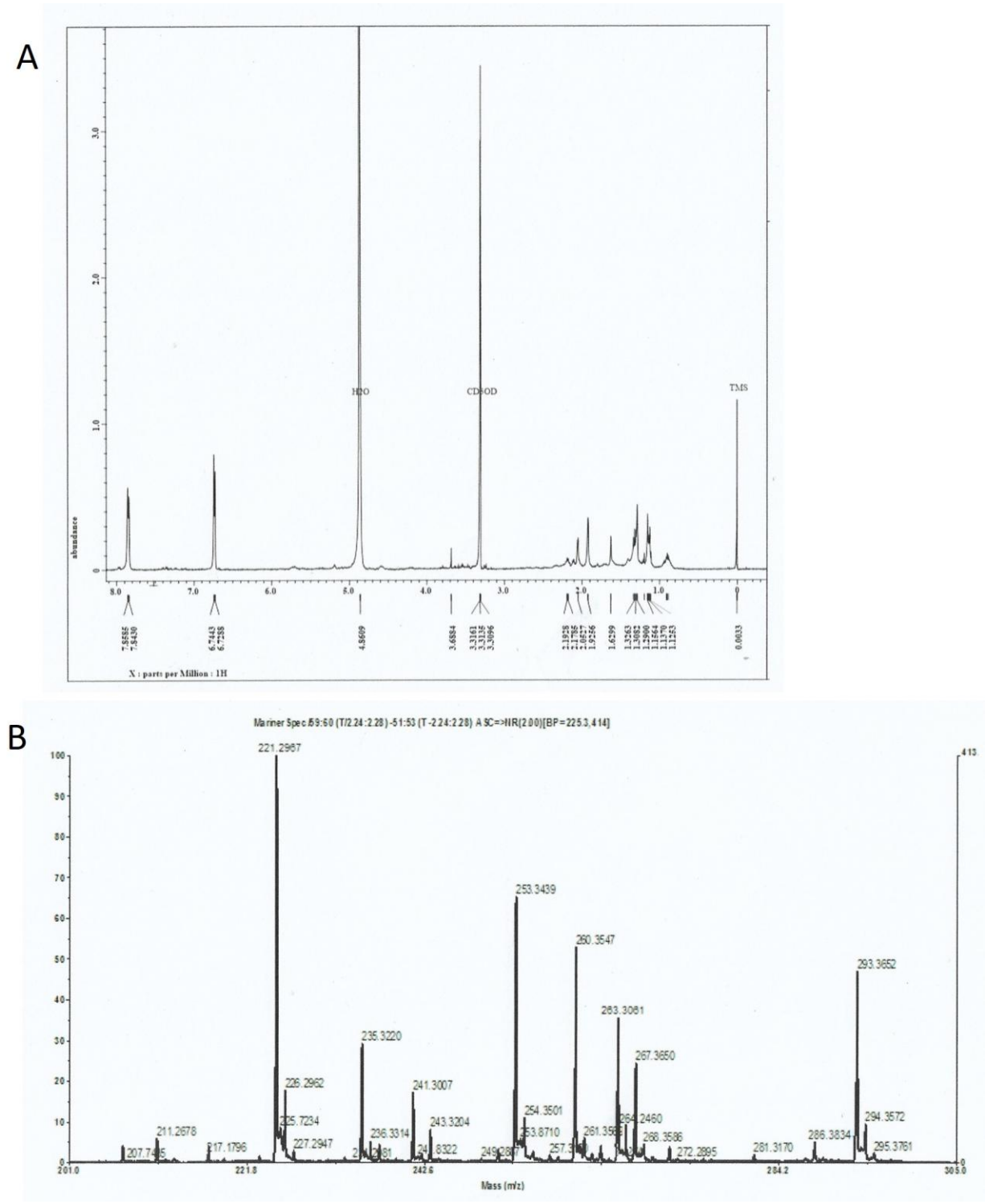

Fig.4:^{1}H-NMR of isolated compound (CD_3OD, 500 MHz) (A); Mass spectrum of isolated compound (ESI-LCMS) (B)

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

^{1H} NMR (δ ppm)	^{13}C NMR (δ ppm)	Predicted functional groups
3.68 (singlet)	177.68 70.12	-C=O ester -OCH3
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) 1.62 (singlet) 1.29, 1.30, 1.32, 1.39 (quartet J = 5.05 Hz) 1.11, 1.12, 1.13, 1.142, 1.149, 1.15 (sextet, J = 5 Hz)	30.67, 35.08, 60.02, 72.04, 100.10	-CH2-
	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 that 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 μg/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 μg/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 µg/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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