Mycobiology

Research Article

Insecticidal Activity of Ethyl Acetate Extracts from Culture Filtrates of Mangrove Fungal Endophytes

Silva Abraham[1], Adi Basukriadi[1], Suyanto Pawiroharsono[2] and Wellyzar Sjamsuridzal[1,*]

[1]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia*
[2]*Center for Bioindustrial Technology, Badan Pengkajian dan Penerapan Teknologi, Jakarta 32344, Indonesia*

Abstract In the search for novel potent fungi-derived bioactive compounds for bioinsecticide applications, crude ethyl acetate culture filtrate extracts from 110 mangrove fungal endophytes were screened for their toxicity. Toxicity tests of all extracts against brine shrimp (*Artemia salina*) larvae were performed. The extracts with the highest toxicity were further examined for insecticidal activity against *Spodoptera litura* larvae and acetylcholinesterase (AChE) inhibition activity. The results showed that the extracts of five isolates exhibited the highest toxicity to brine shrimp at 50% lethal concentration (LC_{50}) values of 7.45 to 10.24 ppm. These five fungal isolates that obtained from *Rhizophora mucronata* were identified based on sequence data analysis of the internal transcribed spacer region of rDNA as *Aspergillus oryzae* (strain BPPTCC 6036), *Emericella nidulans* (strains BPPTCC 6035 and BPPTCC 6038), *A. tamarii* (strain BPPTCC 6037), and *A. versicolor* (strain BPPTCC 6039). The mean percentage of *S. litura* larval mortality following topical application of the five extracts ranged from 16.7% to 43.3%. In the AChE inhibition assay, the inhibition rates of the five extracts ranged from 40.7% to 48.9%, while eserine (positive control) had an inhibition rate of 96.8%, at a concentration of 100 ppm. The extracts used were crude extracts, so their potential as sources of AChE inhibition compounds makes them likely candidates as neurotoxins. The high-performance liquid chromatography profiles of the five extracts differed, indicating variations in their chemical constituents. This study highlights the potential of culture filtrate ethyl acetate extracts of mangrove fungal endophytes as a source of new potential bioactive compounds for bioinsecticide applications.

Keywords *Artemia salina*, Ethyl acetate extracts, Insecticidal activity, Mangrove fungal endophytes, *Spodoptera litura*

Safety and environmental issues surrounding the use of chemical insecticides have led to the development of alternative insect control measures, including bioinsecticides. A bioinsecticide is a formulation of naturally occurring substances that control pests through nontoxic mechanisms and in an ecofriendly manner. They can be animal, plant, or microorganism derived, and exploit living organisms (natural enemies), their products (phytochemicals, microbial products), and their byproducts (semiochemicals) in the management of insects [1]. While the marine environment is considered as a prolific resource for the isolation of less frequently exploited microorganisms [2], recent studies have focused mainly on the application of marine microorganisms in human drug development, with limited information regarding their insecticidal activities having been reported to date [2].

Mangrove fungi, a well-known marine-derived source of bioactive compounds, have recently been used in the isolation of new chemical compounds [3]; the mangrove ecosystem exhibits unique conditions that influence the fungal metabolite. Mangrove-associated fungi provide a broad variety of bioactive secondary metabolites with unique structures, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, and xanthones, among others [3]. The symbiotic nature of endophytic microorganisms favors metabolic interactions with their host plants and their environment, thereby increasing the production of bioactive compounds [4]. In particular, it has been demonstrated that the bioactive compounds produced by endophytic microorganisms contribute to natural plant defenses by preventing herbivory and invasion from superficial pathogens [4]. Several natural products (e.g., microbial polyketides) derived from microorganisms, such as avermectins and milbemycins, have been reported as potent insecticides against various insects and parasites [2]. In addition, they are believed to be the biggest selling and, arguably, most effective acaricides

Mycobiology 2015 June, **43**(2): 137-149
http://dx.doi.org/10.5941/MYCO.2015.43.2.137
pISSN 1229-8093 • eISSN 2092-9323

*Corresponding author
E-mail: sjwelly@hotmail.com

Received March 4, 2015
Revised March 17, 2015
Accepted April 8, 2015

and anthelmintics currently available [2]. Nevertheless, the use of fungal secondary metabolites as bioinsecticides remains limited; most secondary metabolite-based bioinsecticides are dominated by bacteria [5].

Mangrove plants, such as *Rhizophora apiculata*, *Rhizophora annamalayana*, and *Rhizophora mucronata*, have been identified as potential sources of fungal isolates [6-11]. Many studies have explored the active compounds from mangrove fungal secondary metabolites for pharmaceutical applications, but research regarding their agrochemical applications remains scarce [12]. In Indonesia, mangrove fungal research has also focused on the exploration of mangrove fungal secondary metabolites for pharmaceutical applications [9-11, 13], especially antibiotics; however, no research on their agrochemical applications has been reported to date. Many mangrove fungi isolated from mangrove species or genera have been used to extract biologically active compounds, several of which have exhibited insecticidal activities [14-16]. Recently, new active compounds, including 6-hydroxy-3-methylisochroman-5-carboxylic acid, mycoepoxydiene, 5-carboxylmellein, 5-methylmellein, pyrrole-2-carboxylic acid, and 3-methylhydantoin, were isolated from mangrove fungal culture media and shown to exhibit insecticidal activity against *Helicoverpa armigera* and *Sinergasilus* sp. [17].

In Indonesia, one of the countries with the highest diversity of mangrove plants [18, 19], there has been a limited number of studies on the prospecting of mangrove fungi for agrochemical applications. In an attempt to discover a novel substance of insecticidal importance against agricultural insects, the present study evaluates the cytotoxicity and insecticidal activities of ethyl acetate extracts from culture filtrates of endophytic fungal isolates obtained from the Indonesian mangrove plants *R. mucronata*, *Sonneratia alba*, and *Avicennia marina*.

MATERIALS AND METHODS

Plant samples, fungal isolates, and test organisms. Plant samples were collected from the Prof. Dr. Sedyatmo Angke Kapuk Mangrove Rehabilitation and Ecotourism, Jakarta, Indonesia (106°75′48.09″ N, 6°12′19.51″ E), by Silva Abraham on August 14, 2012. Samples of healthy mature living leaves, twigs, roots, and leaves litter from one mature tree of three mangrove plants (*R. mucronata*, *S. alba*, and *A. marina*) were chosen at random. Those samples were brought to the lab in sterile bags and processes within a few hours after sampling.

Fungal isolates obtained from the plant samples were deposited in the Badan Pengkajian dan Penerapan Teknologi Culture Collection (BPPTCC), Tangerang, Indonesia. Fungal isolates were preserved in a 2 mL CryoTube containing 1 mL of 10% glycerol solution (v/v) and 5% lactose (w/v) at −80°C.

Brine shrimp (*Artemia salina*) used for the lethality bioassay were hatched from brine shrimp eggs (Pfizer Consumer Inc., New York, NY, USA). One gram of *A. salina* eggs were hatched in 1,000 mL of artificial seawater [20] with air bubbling and artificial illumination for 36 hr [21]. The phototropic nauplii (larvae) were collected with a pipette from the lighted side and concentrated in a 7-mL test tube. *Spodoptera litura* larvae were obtained from laboratory colonies maintained by the Center for Bioindustrial Technology, Badan Pengkajian dan Penerapan Teknologi, Tangerang, Indonesia.

Fungal isolation. The plant samples (twigs, roots, leaves, and leaf litter) were washed with sterile artificial seawater [20], then surface sterilized using the method described by Ananda and Sridhar [22]. All of the plant samples were placed in sterile tissue paper and dried in laminar airflow for 24 hr. The effectiveness of the sterilization procedure was evaluated following the method developed by Schulz *et al.* [23]. Briefly, sterilized tissue segments (approximately 1 cm × 1 cm) were pressed onto the surface of potato dextrose agar medium (Merck, Whitehouse Station, NJ, USA). The absence of growth of any fungi on the medium, other than endophytic fungi from the internal tissue of the plant samples confirmed that the sterilization procedure was effective in removing the surface fungi [23].

A combination of five isolation methods, namely direct plating [22], filtration using Whatman filter paper [24], filtration using a Millipore membrane [24], particle washing [25], and a moist chamber [26], were used to isolate the endophytic fungi. Further, six media were used for the isolation, namely *Beauveria* spp. isolation medium [27], a modification of the Tubaki medium (1 g peptone, 1 g KH_2PO_4, 0.5 g yeast extract, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.02 g $FeSO_4$, 2 g agar, 500 mL artificial seawater) [28], *Metarhizium* spp. isolation medium [28], chitin medium [29], extract of mangrove leaf medium (following the method of Delalibera *et al.* [30]), and LC Miura's agar medium [31]. Antibiotics, 0.5 mL from 0.6 g/mL streptomycin (Kimia Farma, Jakarta, Indonesia), 0.5 mL from 0.05 g/mL tetracycline (Kimia Farma), 0.5 mL from 0.1 g/mL dodine (Sigma-Aldrich, St. Louis, MO, USA), and 2.5 mL from 0.05 g/mL cycloheximide (Sigma-Aldrich) [27], were added to each of the isolation media. All fungal isolates grown on isolation media were purified and preserved in a 2 mL CryoTube containing 1 mL of 10% glycerol solution (v/v) and 5% lactose (w/v) at −80°C.

Fungal identification. The fungal isolates were identified based on sequence data of the internal transcribed spacer (ITS) rDNA (including ITS1, 5.8S rDNA, and ITS2). Nuclear rDNA from the fungal isolates was extracted using a PrepMan Ultra kit (Applied Biosystems, Foster City, CA, USA). The ITS rDNA of the fungal isolates were amplified with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as described by White *et al.* [32]. PCR thermal cycling was carried out in a PCR thermal cycler using the following parameters:

95°C for 1 min, followed by 40 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension cycle of 72°C for 5 min [33]. Purified PCR products were sequenced using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer; Applied Biosystems). The fungal isolates were identified based on sequence homology with fungal sequences obtained from the GenBank DNA database hosted by NCBI (http://blast.ncbi.nlm.nih.gov), using the BLAST search tool. The identification of the isolates was based on the sequence similarity cutoff point for fungal species delimitation of at least ≥ 97% according to Brock *et al.* [34] and with E-value cut-off 0.01. The sequences of ITS rDNA of fungal isolates were aligned with other sequences retrieved from GenBank using ClustalX [35]. Phylogenetic tree were constructed using the neighbor-joining method [36] with bootstrap values based on 1,000 replications [37]. The evolutionary distances were computed using the Kimura 2-parameter method [38]. The sequence data of ITS rDNA of the fungal strains were deposited into GenBank under the following accession numbers: KP165432 (*Emericella nidulans* BPPTCC 6035), KP165433 (*Aspergillus oryzae* BPPTCC 6036), KP165434 (*Aspergillus tamarii* BPPTCC 6037), KP165435 (*E. nidulans* BPPTCC 6038), and KP165436 (*Aspergillus versicolor* BPPTCC 6039).

Fermentation and extraction of culture filtrates. The fungal cell suspension (1 mL) from a seven-day-old culture grown on an malt extract agar (MEA) slant was inoculated into 100 mL Erlenmeyer flasks containing 19 mL of malt extract broth (MEB) medium [20], incubated for seven days on a rotary shaker at 65 rpm at room temperature, and used as a starter culture. This starter culture was then inoculated into a 500-mL Erlenmeyer flask containing 180 mL of fresh MEB medium and incubated for 14 days on a rotary shaker at 65 rpm. The fungal mycelium on each flask was collected using Whatman #1 filter paper, after which the filtrate was extracted with 100 mL of ethyl acetate, using a separation funnel. The water fraction (upper layer) was collected and re-extracted (three times) with ethyl acetate. The ethyl acetate fraction (bottom layer) was collected and evaporated using a rotary evaporator.

Brine shrimp lethality bioassay. A brine shrimp lethality bioassay using larvae of *A. salina* was performed for the preliminary evaluation of toxicity from all fungal extracts. The bioassay was conducted to determine the 50% lethal concentration (LC_{50}) of the fungal culture ethyl acetate extracts. For preliminary screening, five concentrations of culture filtrate crude extracts (1,000, 500, 250, and 125 ppm) were tested against 20 larvae of *A. salina* for each concentration and repeated three times. The ethyl acetate extracts (10 µL) were evaporated in a 7-mL test tube and dissolved in 5 mL of artificial seawater; then, 10 µL of dimethyl sulfoxide (DMSO) were added to completely dissolve the extracts. The negative control consisted of 10 µL of pure ethyl acetate (evaporated) and 10 µL of DMSO in 5 mL of artificial seawater. The mortality rate was recorded after exposing 20 larvae for 24 hr. The concentration of the testing ethyl acetate extract was gradually decreased following recording of the larvae mortality rate. The fungal extracts that exhibited the highest percentage mortality rates (≥ 90%) were chosen as candidates for the next test and tested in a decreased concentration. After a series of tests (decreasing gradually from 1,000 ppm to 500, 250, and 125 ppm), the fungal extracts was tested against *A. salina* larvae to determine LC_{50}. The extract concentrations used in this test were 80, 40, 20, 10, and 5 ppm.

Topical application of culture filtrate extracts to *S. litura* larvae. The larvicidal activity of the fungal ethyl acetate extracts was evaluated using topical application to *S. litura* III instar larvae, as described by Supriyono [39]. Briefly, 5 µg of fungal extract were dissolved in 2 µL of acetone (analytical grade; Merck, Darmstadt, Germany) and applied to the dorsum of *S. litura* III instar larvae using a micropipette. Ten *S. litura* III instar larvae (10 to 14 mg in weight and 1 to 1.3 cm in length) were used for each extract treatment, and each treatment was performed in triplicate. Acetone (2 µL) was used as a negative control and the commercial insecticide deltamethrin (5 µg/2 µL; Decis 25 g/L; PT Bayer, Jakarta, Indonesia) was used as a positive control. Following treatment, the larvae were introduced into a 100-mL plastic cup and covered with cotton sheets. An artificial diet was prepared according to the method of Supriyono [39] for consumption by the treated larvae, consisting of 150 g of soybeans (soaked in 460 mL of distilled water for 24 hr), 3 g of L-(+)-ascorbic acid, 3 g of nipagine (p-hydroxybenzoic acid ethyl ester), 11 g of dried yeast powder, 180 mg of gentamicin sulfate, 1 mL of paraformaldehyde, 10 g of agar, and 315 mL of distilled water. The experiment was performed for 5 days and mortality was assessed on the fifth day. A larva was considered dead if it was unable to make a coordinated movement when gently prodded. The mortality percentage of each treatment was recorded. The larval percentage mortality rate was calculated according to the formula of Ladhari *et al.* [40]: % mortality = (Number of dead larvae/ Total number of larvae) × 100.

Acetylcholinesterase (AChE) inhibition assay. Fungal extracts were evaluated spectrophotometrically for AChE inhibition potential using AChE iodide (AChI; Sigma) as a substrate, following Ellman's assay [41]. Fungal extracts (25 µL; 100 ppm concentration) were added to a 96-well microplate (Axygen BioScience, Inc., Union City, CA, USA) containing 50 µL bovine serum albumin (0.1%; Sigma) and 25 µL of AChE enzyme (0.28 U/mL; Sigma), after which 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB; 125 µL, 3 mM; Sigma) were added to the mixture and incubated for 15 min in a water bath at 30°C. To start the reaction, 25 µL of 15 mM AChI was added to the mixture. The reaction between thiocholine and DTNB was indicated by the

formation of the 5-thio-2-nitrobenzoate anion, indicated by a yellow color appearance. Absorbance was measured using an enzyme-linked immunosorbent assay reader (Elx800; BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 400 nm for 8 min. The fungal extract-free reaction mixture was taken as a negative control and with the standard drug eserine (Sigma) as a positive control. Each assay was performed in triplicate and the AChE inhibitory value was taken as the average of three independent experiments. The percentage of enzyme activity and percentage of inhibition were calculated according to the equation in Ellman's assay [41].

High-performance liquid chromatography (HPLC) analysis of the ethyl acetate extracts from the fungal extracts. The active ethyl acetate extracts were analyzed by HPLC (HPLC-UV Vis Detector; Waters Co., Milford, MA, USA). All ethyl acetate extracts were loaded onto a C18 column in a 20-μL injection volume. Elution was performed using a linear gradient consisting of double distilled water and acetonitrile; an isocratic step was initially carried out for 3 min at 85% water, followed by a moderate increase in acetonitrile to reach 100% in 20 min, at a flow rate of 1 mL/min [42]. The second isocratic step was carried out for 5 min with 100% acetonitrile. The elution profiles of the secondary metabolite extracts were observed using absorption at 254 nm.

Statistical analyses. One-way analysis of variance (ANOVA) was used to compare the treatment means of brine shrimp lethality bioassay, topical application to *S. litura* larvae, and AChE inhibition assay. A *post-hoc* Tukey's honestly significant difference (HSD) test, with a significance level of $\alpha = 0.05$, was performed when a significant difference between treatment means was detected. The mortality rates of brine shrimp and *S. litura* larvae were corrected for control mortality using Abbott's formula [43]. The corrected mortality rates were normalized by an arcsine square-root transformation before the ANOVA test was conducted. The concentration at which 50% of brine shrimp died (LC_{50}) for each fungal extract was determined by probit analysis [44]. All statistical analyses were performed using IBM SPSS Statistics ver. 21 software (IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

Preliminary lethality test of ethyl acetate extracts from fungal culture filtrates. To investigate the toxicity of 110 ethyl acetate extracts produced by 110 fungal culture filtrates (hereinafter referred to as extracts), a brine shrimp (*A. salina*) lethality test was used as the preliminary screening method. According to Harwig and Scott [21], extracts that cause more than 90% larval mortality are rated as highly toxic. As shown in Table 1, the serial decrease in concentration of extracts from 1,000 to 500, 250, and 125 ppm was followed by decreased in number of extracts of fungal isolates that exhibited toxic activities that cause more than 90% larval mortality. Table 1 highlighted only five extracts that displayed the highest toxicity at a concentration of 125 ppm.

Table 1. Preliminary brine shrimp (using larvae of *Artemia salina*) lethality bioassay of 110 ethyl acetate extracts from 110 fungal isolates at serially decreasing concentrations

No.	Extract concentration (ppm)	No. of extracts that exhibited toxic activities (mortality ≥ 90%)
1	1,000	39
2	500	30
3	250	8
4	125	5

Identification of five fungal isolates that produced toxic extracts. The results of the sequence analysis by the BLAST homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) based on the sequence data of ITS rDNA of the five fungal isolates that produced toxic extracts indicated a high homology with their closely related species, as obtained from GenBank (99%~100%). According to Brock *et al.* [34], the homology of ITS rDNA sequences of organisms belonging to the same species is ≥ 97%. Consequently, based on the sequence similarity cutoff point for fungal species [34], the five fungal isolates were identified as *E. nidulans* (isolate ARM1M1 = strain BPPTCC 6035 and isolate DRM3M3 = BPPTCC 6038); *A. versicolor* (isolate SRM6T4 = strain BPPTCC 6039); *A. oryzae* (isolate ARM3L1 = strain BPPTCC 6036); and *A. tamarii* (isolate DRM2KS1 = strain BPPTCC 6037). The phylogenetic tree (Fig. 1) shows that the position of each fungal strain used in this study is closely related with that of other strains from the same species retrieved from GenBank; they are clustered together, with high bootstrap support (86%~98%). As shown in Fig. 1, *A. versicolor* BPPTCC 6039 isolated from *R. mucronata* litter is closely related to four *A. versicolor* strains from GenBank, with very strong bootstrap support (98%). Two strains of *E. nidulans* isolated from roots and leaves of *R. mucronata* are clustered together with type strain *E. nidulans* and two strains from GenBank, with strong support (86%). *A. tamarii* BPPTCC 6037, isolated from leaves of *R. mucronata*, is located within the same cluster as two strains of *A. tamarii* from GenBank, with very strong bootstrap support (99%). *A. oryzae* BPPTCC 6036, isolated from roots of *R. mucronata*, is clustered together with type strain *A. oryzae* and other strains from GenBank, with strong bootstrap support (88%). The list of fungal strains, their origins, and their accession numbers of ITS rDNA sequence data used to draw the phylogenetic tree are shown in Table 2. As shown in Table 2, *Aspergillus* strains retrieved from GenBank were isolated from various environments, including soil, metal corrosion, milk, and rice wine starter. *Aspergillus* is a cosmopolitan fungus found in diverse environments and

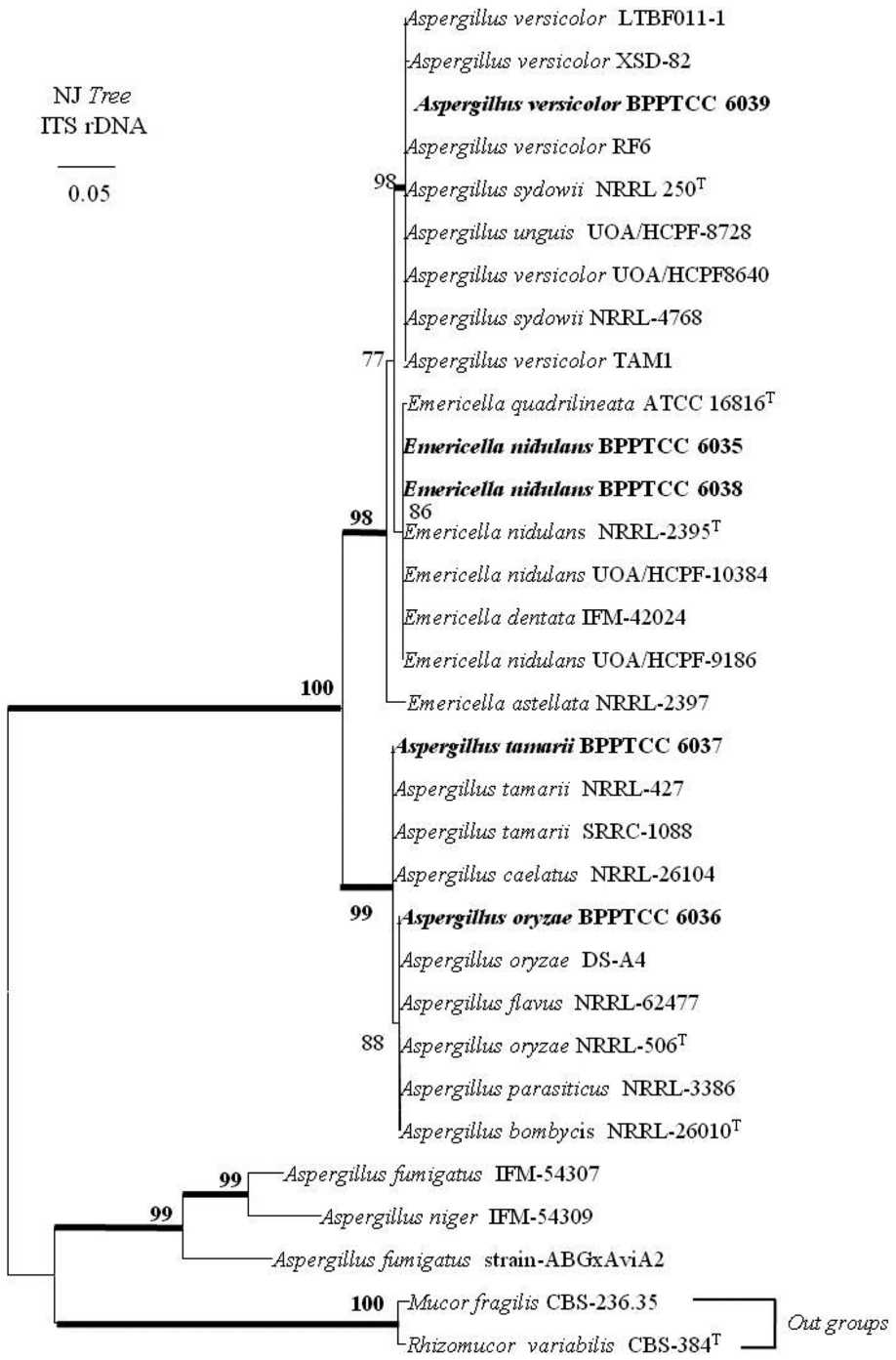

Fig. 1. Phylogenetic position of mangrove fungal endophytes based on internal transcribed spacer rDNA sequence data. Strains used in this study are indicated in bold.

a wide range of habitats [45]. In marine and estuarine environments, *Aspergillus* is one of several fungal genera often found in different hosts, from marine organisms to mangrove plants [3, 28]. *Aspergillus* species (*A. flavus*, *A. niger*, and *A. nidulans*) have been isolated from leaves, twigs, and roots of several *Rhizophoraceae* mangroves such as *R. mucronata*, *R. stylosa*, and *R. apiculata* [14, 46]. It has been reported that *Aspergillus* fungi from those mangrove species produce a wide variety of bioactive secondary metabolites that display activities against human microbial pathogens and cancer Hep2 and MCF7 cell lines [47]. Other fungal species that have been isolated from *Rhizophora* are *Phomopsis* spp. from *R. apiculata* [6], *Meyerozyma guilliermondii* from the leaves of *R. mucronata* [48], *Fusarium oxysporum* from *R. annamalayana* [49], and two species of *Pestalotiopsis* from the twigs of two mangrove plants, *R. apiculata* and *R. mucronata* [7]; these fungal species have also produced secondary metabolites with

Table 2. Strains and their accession numbers of ITS rDNA sequences data used to draw the phylogenetic tree

No.	Fungal strain	Accession No.	Sources of strain
1	*Aspergillus versicolor* LTBF011-1	GQ229082	Biocorrosion on metallic surfaces
2	*Aspergillus versicolor* XSD-82	EU326209	Soil
3	*Aspergillus versicolor* RF6	GU232767	Endophytic fungi
4	*Aspergillus sydowii* NRRL 250^{T}	AY373868	Type strain
5	*Aspergillus unguis* UOA/HCPF-8728	FJ878626	Immunocompromised host
6	*Aspergillus versicolor* UOA/HCPF8640	FJ878625	Immunocompromised hosts
7	*Aspergillus sydowii* NRRL-4768	EF652473	Soil, California
8	*Aspergillus versicolor* TAM1	JN997427	Spot on ancient book
9	*Emericella quadrilineata* ATCC 16816^{T}	AY373889	Type strain (soil, New Jersey)
10	*Emericella nidulans* NRRL-2395^{T}	AY373888	Soil, Argentina
11	*Emericella nidulans* UOA/HCPF-10384	FJ878647	Immunocompromised hosts
12	*Emericella dentata* IFM-42024	AB248999	Unknown
13	*Emericella nidulans* UOA/HCPF-9186	FJ878641	Immunocompromised hosts
14	*Emericella astellata* NRRL-2397	EF652447	Dead plant leaf, Seymour Island, Galapagos, Ecuador
15	*Aspergillus tamarii* NRRL-427	HQ340111	Tomato
16	*Aspergillus tamarii* SRRC-1088	AY373870	Cotton seed, Tennessee
17	*Aspergillus caelatus* NRRL-26104	AF272575	Peanut field soil, 2.5 km east of Herod, Terrell County, GA
18	*Aspergillus oryzae* DS-A4	HQ285552	Traditional starter cultures (nuruks) used for rice wine in Korea
19	*Aspergillus flavus* NRRL-62477	JX292092	Paprika
20	*Aspergillus oryzae* NRRL-506^{T}	AF459735	Milk, VT
21	*Aspergillus parasiticus* NRRL-3386	HQ340110	-
22	*Aspergillus bombyc*is NRRL-26010^{T}	AF104444	Silk worm excrement, Japan
23	*Aspergillus fumigatus* IFM-54307	AB363746	Soil, Japan
24	*Aspergillus niger* IFM-54309	AB363747	Soil, Japan
25	*Aspergillus fumigatus* strain-ABGxAviA2	KF297885	Digestive tract of termite *Reticulitermes santonensis*
26	***Emericella nidulans* BPPTCC 6035**	**KP265432**	**Root of *R. mucronata*, Indonesia (this study)**
27	***Aspergillus oryzae* BPPTCC 6036**	**KP265433**	**Root of *R. mucronata*, Indonesia (this study)**
28	***Aspergillus tamarii* BPPTCC 6037**	**KP265434**	**Leaf of *R. mucronata*, Indonesia (this study)**
29	***Emericella nidulans* BPPTCC 6038**	**KP265435**	**Leaf of *R. mucronata*, Indonesia (this study)**
30	***Aspergillus versicolor* BPPTCC 6039**	**KP265436**	**Leaf litter of *R. mucronata*, Indonesia (this study)**
31	*Mucor fragilis* CBS-236.35	JN206422	Fungus, *Tremella* sp.
32	*Rhizomucor variabilis* CBS-384^{T}	HM849679	Face, China

Nucleotide sequences determined in this study are indicated in bold.

ITS, internal transcribed spacer; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL; UOA/HCPF, University of Athens/Hellenic Collection of Pathogenic Fungi, National and Kapodistrian University of Athens; ATCC, American Type Culture Collection, Manassas, VA; IFM, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japanese Federation of Culture Collections; SRRC, Southern Regional Research Center, Agricultural Research Service, US Department of Agriculture, New Orleans, LA; BPPTCC, Badan Pengkajian dan Penerapan Teknologi (Agency for The Assessment and Applications of Technology) Culture Collection, Tangerang, Indonesia; CBS, Centraalbureau voor Schimmelcultures, Utrecht; other abbreviations are not registered.

various activities. Other *Aspergillus* isolated from various mangrove species include *A. flavipes*, from the inner leaves of *Acanthus ilicifolius* [50], *Aspergillus* sp., from the leaves of *Ipomoea pes-caprae* [51], *A. flavus*, from the roots of *Hibiscus tiliaceus* [52], and nine *Aspergillus* species, namely *A. niger*, *A. fumigatus*, *A. aureoles*, *A. candidus*, *A. chevalier*, *A. flavus*, *A. glaucus*, *A. ochraceus*, and *A. smithii*, from decomposing leaves of *Ceriops tagal* [53].

Brine shrimp lethality bioassay of five extracts from fungal culture filtrates. The brine shrimp lethality bioassay of the five selected fungal extracts at lower concentrations (2.5, 5, 10, 20, 40, and 80 ppm) showed that, as the extract concentration increased, so did the degree of toxicity. All five extracts exhibited a similar pattern. One-way ANOVA showed that different concentrations of the extracts from *A. oryzae* [$F(5, 12) = 45.69$, $p < 0.001$], *E. nidulans* BPPTCC 6035 [$F(5, 12) = 66.86$, $p < 0.001$], *E. nidulans* BPPTCC 6038 [$F(5, 12) = 72.71$, $p < 0.001$], *A. tamari* [$F(5, 12) = 22.16$, $p < 0.001$], and *A. versicolor* [$F(5, 12) = 43.17$, $p < 0.001$] had significant effects on the mortality rates of brine shrimp larvae. *Post-hoc* comparisons using Tukey's HSD test indicated significant differences between the means of some pairs of concentrations but not the means of other pairs. Consequently, the mean mortality rates of the brine shrimp bioassay for the five extracts using six concentrations resulted in a different number of homogeneous subsets of means. So, the toxicity rating of

Table 3. Toxicity of ethyl acetate extracts from five fungal strains, based on brine shrimp lethality test using larvae of *Artemia salina*

Fungal strain	Extract concentration (ppm)	Mean ± SE of percentage (%) of death larvae[a]	Toxicity rating[b]
Aspergillus oryzae BPPTCC 6036	2.5	35.0 ± 2.9 a	Slightly toxic
	5.0	41.7 ± 1.7 a	Slightly toxic
	10.0	45.0 ± 5.8 a	Slightly toxic
	20.0	83.3 ± 4.4 b	Toxic
	40.0	96.7 ± 1.7 bc	Highly toxic
	80.0	98.3 ± 1.7 c	Highly toxic
Emericella nidulans BPPTCC 6035	2.5	26.7 ± 1.7 a	Slightly toxic
	5.0	38.3 ± 1.7 b	Slightly toxic
	10.0	48.3 ± 1.7 bc	Slightly toxic
	20.0	56.7 ± 1.7 c	Toxic
	40.0	70.0 ± 2.9 d	Toxic
	80.0	75.0 ± 2.9 d	Toxic
Emericella nidulans BPPTCC 6038	2.5	21.7 ± 1.7 a	Slightly toxic
	5.0	33.3 ± 1.7 ab	Slightly toxic
	10.0	41.7 ± 3.3 b	Slightly toxic
	20.0	71.7 ± 4.4 c	Toxic
	40.0	81.7 ± 3.3 c	Toxic
	80.0	98.3 ± 1.7 d	Highly toxic
Aspergillus tamarii BPPTCC 6037	2.5	30.0 ± 2.9 a	Slightly toxic
	5.0	35.0 ± 5.8 a	Slightly toxic
	10.0	48.3 ± 1.7 ab	Slightly toxic
	20.0	75.0 ± 2.9 bc	Toxic
	40.0	90.0 ± 5.8 bcd	Highly toxic
	80.0	96.7 ± 3.3 d	Highly toxic
Aspergillus versicolor BPPTCC 6039	2.5	25.0 ± 2.9 a	Slightly toxic
	5.0	33.3 ± 1.7 ab	Slightly toxic
	10.0	53.3 ± 1.7 bc	Toxic
	20.0	65.0 ± 5.0 cd	Toxic
	40.0	85.0 ± 2.9 de	Toxic
	80.0	95.0 ± 2.9 e	Highly toxic

BPPTCC, Badan Pengkajian dan Penerapan Teknologi (Agency for The Assessment and Applications of Technology) Culture Collection, Tangerang, Indonesia.

[a]Average of three replicates, standard error (SE); means within the column of each fungal extract followed by the same letter are not significantly different in Tukey's honestly significant difference means comparisons test at $\alpha = 0.05$.

[b]Toxicity of extracts are rated following Harwig and Scott [21]: non-toxic (0%~9%); slightly toxic (10%~49%); toxic (50%~89%) and highly toxic (90%~100%).

each extract corresponded to its subset of means. The rating of the toxicity of the five extracts is shown in Table 3. Of the five extracts tested, four extracts (produced by *A. oryzae* BPPTCC 6036, *E. nidulans* BPPTCC 6038, *A. tamari* BPPTCC 6037, and *A. versicolor* BPPTCC 6039) were rated as highly toxic, and one extract (produced by *E. nidulans* BPPTCC 6035) was rated as toxic at a concentration of 80 ppm. Two extracts (produced by *A. oryzae* BPPTCC 6036 and *A. tamari* BPPTCC 6037) were even highly toxic at concentrations of 40 ppm. Harwig and Scott [21] rated the toxicity of filtrates and extracts based on the mortality rate against *A. salina* larvae as follows: non-toxic (0%~9%), slightly toxic (10%~49%), toxic (50%~89%), and very/highly toxic (90%~100%). The mortality rates of the five fungal extracts in this experiment ranged from 75% to 98.3% at a concentration of 80 ppm. Qiao *et al.* [54] reported that six indoloditerpene derivatives from the endophytic fungus *A. oryzae*, which were isolated from the marine red algae *Heterosiphonia japonica*, displayed a mortality rate of 31.4%~74.2% against *A. salina* at a concentration of 100 μg/mL (0.1 ppm).

LC_{50} values of the ethyl acetate extracts from five fungal strains. Table 4 shows the LC_{50} values of the ethyl acetate extracts from five fungal strains based on the brine shrimp lethality bioassay. The linear regression of the probit analysis was used to calculate the LC_{50} value for each extract, as depicted in Fig. 2. As shown in Table 4, the LC_{50} values of the five fungal extracts against *A. salina* larvae ranged from 7.45 ppm to 10.24 ppm. The extract that showed the lowest LC_{50} value was from *A. tamarii* (7.45 ppm) which, as mentioned, was rated as highly toxic,

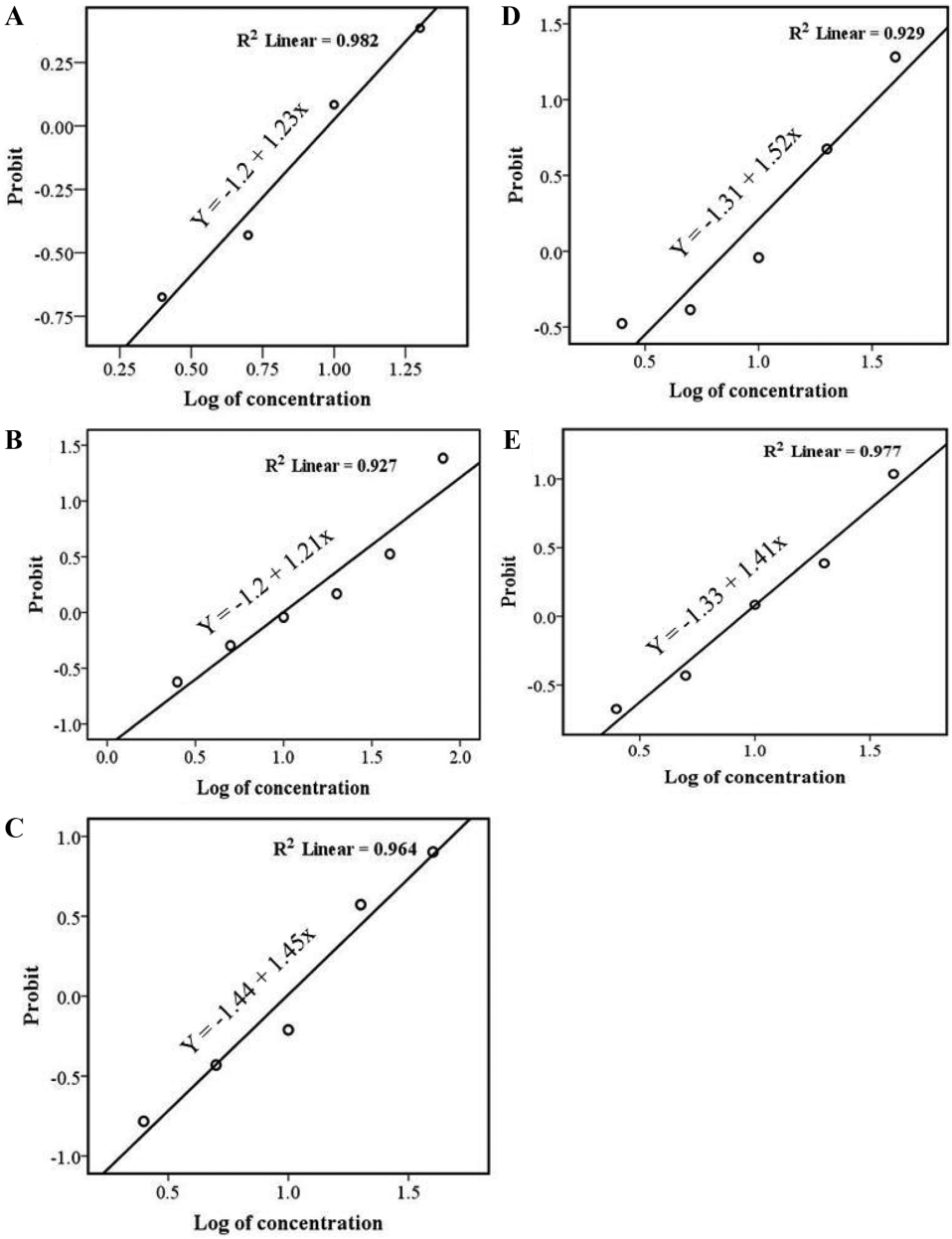

Fig. 2. Linear regression of PROBIT mortality against log concentration of ethyl acetate extracts of culture filtrate from *Aspergillus oryzae* BPPTCC 6036 (A), *Emericella nidulans* BPPTCC 6035 (B), *Emericella nidulans* BPPTCC 6038 (C), *Aspergillus tamarii* BPPTCC 6037 (D), and *Aspergillus versicolor* BPPTCC 6039 (E), based on brine shrimp lethality test using larvae of *A. salina*. BPPTCC, Badan Pengkajian dan Penerapan Teknologi (Agency for The Assessment and Applications of Technology) Culture Collection, Tangerang, Indonesia.

Table 4. LC_{50} values of ethyl acetate extracts from five fungal strains, based on brine shrimp lethality test using larvae of *Artemia salina*

Fungal strain	LC_{50} (ppm)	Regression equation	r^2 regression
Aspergillus oryzae BPPTCC 6036	9.25	y = −1.2 + 1.23x	0.98
Emericella nidulans BPPTCC 6035	10.24	y = −1.2 + 1.21x	0.93
Emericella nidulans BPPTCC 6038	9.96	y = −1.44 + 1.45x	0.96
Aspergillus tamarii BPPTCC 6037	7.45	y = −1.31 + 1.52x	0.93
Aspergillus versicolor BPPTCC 6039	8.89	y = −1.33 + 1.41x	0.98

BPPTCC, Badan Pengkajian dan Penerapan Teknologi (Agency for The Assessment and Applications of Technology) Culture Collection, Tangerang, Indonesia.

causing 96.67% mortality of *A. salina* larvae. Miao *et al.* [55] found that the LC_{50} value of 6-O-methylaverufin produced by the endophytic fungus *A. versicolor* and isolated from the brown algae *Sargassum thunbergii* was 0.5 μg/mL, or 5×10^{-4} ppm. Another study reported that 4-phenyl-3,4-dihydroquinolone and aflaquinolone A derivatives produced by *A. nidulans* which was isolated from fresh leaves of the mangrove plant *Rhizophora stylosa* exhibited toxic activities against *A. salina*, with LC_{50} values ranging from 4.5 to 7.1 μM [46]. In the present study, the LC_{50} values of *A. versicolor* and *E. nidulans* extracts were 8.89 and 9.96 ppm, respectively, more toxic than the results of the two studies discussed [46, 55]. Of note, their studies used single compounds, whereas the present study used crude extracts. According to the standard which classifies active plant compounds and extracts as toxic if their LC_{50} value is < 1,000 μg/mL or < 1,000 ppm [56], all extracts produced by the five fungal strains in this study are classified as toxic.

Topical application of the five extracts onto *S. litura* III instar larvae. The results of the topical application of the five extracts onto *S. litura* III instar larvae (Table 5) indicate that the extract produced by *A. versicolor* resulted in the highest mortality rate of *S. litura* larvae (43.3%) at a 5 μg/mL concentration. The percentage mortality caused by the extract is higher than that of the positive control, the commercial insecticide deltamethrin (36.7%). However, an ANOVA of the larval mortality rates found no significant difference among the treatment means [($F(5, 12) = 2.34$, $p = 0.106$]. Using a topical application bioassay, several studies have demonstrated that *Aspergillus* exhibits insecticidal activity against *S. litura* larvae [57, 58]. The 4-(*N*-methyl-*N*-phenyl amino)-butan-2-one compound produced by *A. gorakhpurensis* yielded a 50% mortality rate of *S. litura* IV instar larvae at a concentration of 330.69 μg/mL [57]. Another study also has reported that the kojic acid produced by *A. funiculosus* resulted in a 50% mortality rate of *S. litura* IV instar larvae at a concentration of 693.52 μg/mL [58]. In the search for new insecticides, acute toxic effects as demonstrated by topical application are usually the yardstick by which products are measured, yielding a spectrum of fast-acting, potent products [59]. The results of the topical application bioassay in this study indicate that the five extracts tested have acute toxic effects on *S. litura* larvae. As well, the degree of acute toxicity of the five extracts in this study is higher than reported in the two earlier studies [57, 58]. This finding suggests that the five extracts have high potential as source of active compounds for bioinsecticides.

Table 5. Percentage of larval mortality from topical application bioassay (5 μg/mL) of five fungal extracts on *Spodoptera litura* instar III larvae

Fungal strain	Percentage mortality (mean ± SE)[a]
Emericella nidulans BPPTCC 6038	16.7 ± 8.8
Emericella nidulans BPPTCC 6035	16.7 ± 3.3
Aspergillus oryzae BPPTCC 6036	20.0 ± 3.3
Aspergillus tamarii BPPTCC 6037	16.7 ± 3.3
Aspergillus versicolor BPPTCC 6039	43.3 ± 14.5
Positive control (deltamethrin)	36.7 ± 6.7

BPPTCC, Badan Pengkajian dan Penerapan Teknologi (Agency for The Assessment and Applications of Technology) Culture Collection, Tangerang, Indonesia.

[a]Average of three replicates, standard error (SE). There is no significant difference of means ($p = 0.106$) by one way ANOVA.

Table 6. Percentage of acetylcholinesterase (AChE) inhibition potential of five fungal extracts at concentration of 100 ppm

Fungal strain	Percentage of AChE (mean ± SE)[a]
Emericella nidulans BPPTCC 6038	47.6 ± 0.6 ab
Aspergillus versicolor BPPTCC 6039	46.4 ± 0.1 a
Aspergillus tamarii BPPTCC 6037	48.9 ± 0.5 b
Emericella nidulans BPPTCC 6035	40.8 ± 0.2 c
Aspergillus oryzae BPPTCC 6036	40.7 ± 0.3 c
Eserine (positive control)	96.8 ± 0.3 d

BPPTCC, Badan Pengkajian dan Penerapan Teknologi (Agency for The Assessment and Applications of Technology) Culture Collection, Tangerang, Indonesia.

[a]Average of three replicates, standard error (SE); means followed by the same letter are not significantly different in Tukey's honestly significant difference means comparisons test at $\alpha = 0.05$.

The five extracts ranged from 40.7% to 48.9%, while eserine had an inhibition rate of 96.8%. ANOVA found a highly significant difference in the inhibition rates of the extracts and eserine [$F(5, 12) = 3{,}414.51$, $p < 0.001$]. A *post-hoc* Tukey's HSD test showed that eserine had significantly higher AChE inhibitory activity than all the extracts. Among the five extracts, the extract produced by *A. tamarii* BPPTCC 6037 had the highest inhibition rate (48.9%), though it did not differ significantly from the extract produced by *E. nidulans* BPPTCC 6038 (47.6%). This study showed that the crude extracts produced by *A. tamarii* BPPTCC 6037, *E. nidulans* BPPTCC 6038, and *A. versicolor* BPPTCC 6039 exhibited a high AChE inhibitory activity at a concentration of 100 ppm. The extracts used were crude extracts, so their potential as sources of AChE inhibition compounds makes them likely candidates as neurotoxins. In a previous study, Qiao *et al.* [54] reported that the AChE inhibitory activity of six indoloditerpene derivatives produced by the endophytic fungus *A. oryzae* ranged from 4% to 16.4% at a concentration of 100 μg/mL (0.1 ppm).

HPLC analysis of the extracts produced by five fungal strains. The HPLC profiles of the ethyl acetate extracts produced by five fungal strains (Fig. 3) show that each

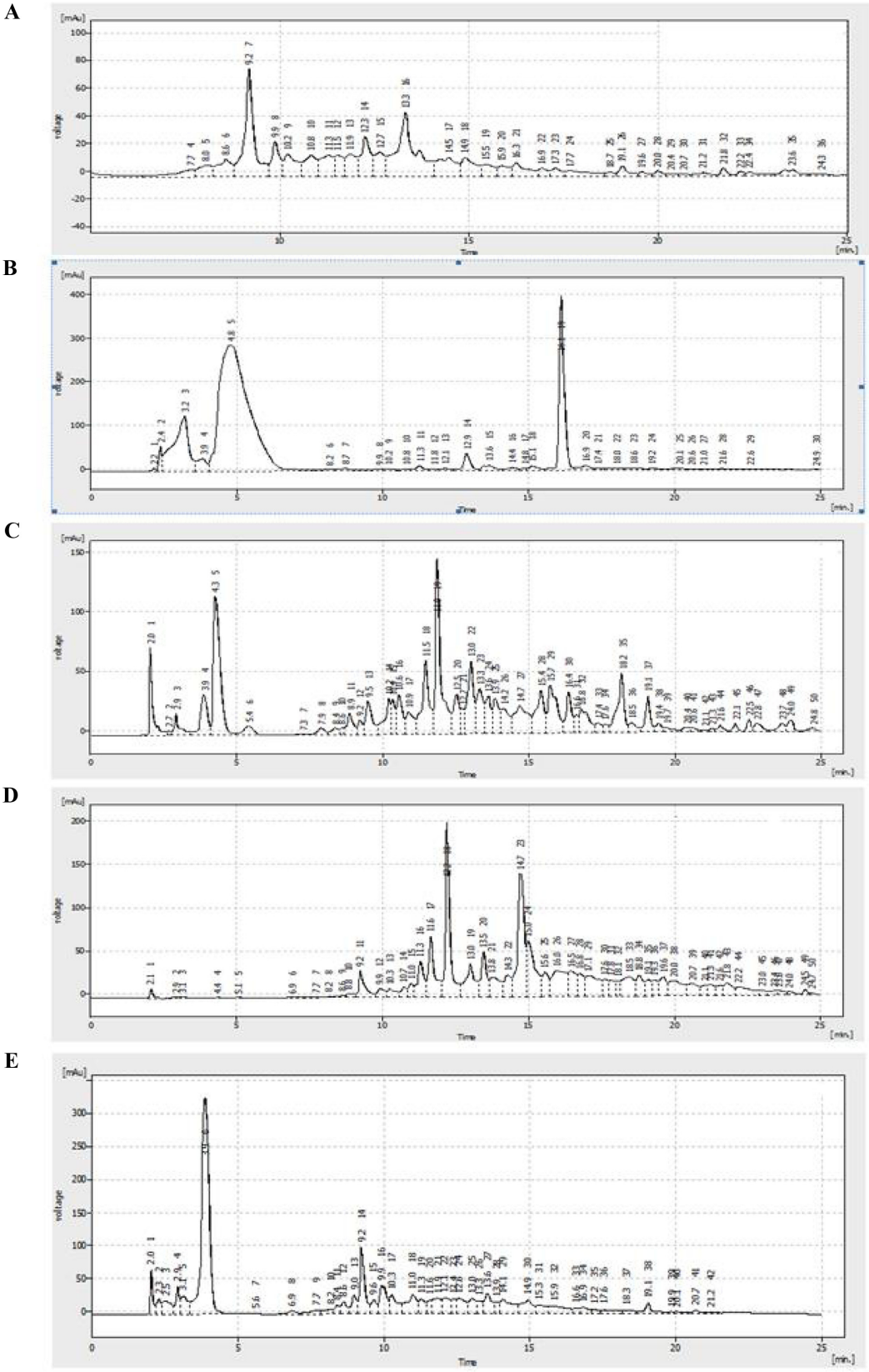

Fig. 3. High-performance liquid chromatography profiles from ethyl acetate extracts of culture filtrates produced by *Aspergillus oryzae* BPPTCC 6036 (A), *Emericella nidulans* BPPTCC 6035 (B), *Emericella nidulans* BPPTCC 6038 (C), *Aspergillus tamarii* BPPTCC 6037 (D), and *Aspergillus versicolor* BPPTCC 6039 (E). BPPTCC, Badan Pengkajian dan Penerapan Teknologi (Agency for The Assessment and Applications of Technology) Culture Collection, Tangerang, Indonesia.

extract has a different peak characteristic profile. The results of the HPLC analysis of the extracts indicate that the five extracts are composed of different compounds. The profiles indicated differences between the chemical constituents and the secondary metabolites. Further studies, including fractionation, isolation, and characterization of each peak, are therefore required since each ethyl acetate extract peak might represent a single compound with a different toxicity. Additionally, assessing the toxicity degree of each fraction and elucidating the best toxic fraction may lead to the discovery of new active compounds. Through optimization of the fermentation and extraction process, the toxic fraction could be increased, consequently increasing the degree of toxicity, acute toxicity, and AChE inhibition activity of extracts.

In the development of bioinsecticidal compounds, complex mixtures are likely to be more durable in terms of insects developing resistance and behavioral desensitization [59]. According to Hummelbrunner and Isman [59], a complex mixture of bioinsecticidal compounds usually has a synergistic effect; compounds lacking acute toxicity might still confer protection to crops by reducing the fitness of insect herbivores through the inhibition of larval growth, disruption of larval development, or failure in pupal eclosion. The complex mixture of compounds or crude extracts presented herein exhibited toxicity, acute toxicity, and neurotoxicity, demonstrating the synergistic effect required for bioinsecticidal development.

In summary, the ethyl acetate extracts of culture filtrates from five mangrove endophytic fungi exhibited high toxicities against brine shrimp and *S. litura* larvae as well as AChE inhibitors. The HPLC analysis demonstrated that the extracts had different profiles and peak characteristics. Thus, the extracts of culture filtrates of mangrove endophytic fungi might provide new bioactive compounds for use as potential bioinsecticides. Further study on the screening and characterization of bioactive compounds from the extracts are warranted.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Ministry of Research and Technology, Republic of Indonesia, for providing financial support in the form of a doctoral program scholarship and research expenses to SA. In addition, we thank the Center of Excellence for Indigenous Biological Resources—Genome Studies (CoE IBR-GS), Faculty of Mathematics and Natural Sciences, Universitas Indonesia, for providing the facilities to perform the identification of isolates studied. We also express our gratitude to Dr. Agus Supriyono, from Badan Pengkajian dan Penerapan Teknologi, for his help in performing HPLC analysis and data interpretation.

REFERENCES

1. Kumar S. Biopesticides: a need for food and environmental safety. J Biofertil Biopestic 2012;3:e107.
2. Arasu MV, Al-Dhabi NA, Saritha V, Duraipandiyan V, Muthukumar C, Kim SJ. Antifeedant, larvicidal and growth inhibitory bioactivities of novel polyketide metabolite isolated from *Streptomyces* sp. AP-123 against *Helicoverpa armigera* and *Spodoptera litura*. BMC Microbiol 2013;13:105.
3. Joel EL, Bhimba BV. Biological activity of secondary metabolites isolated from mangrove fungi *Neurospora crassa*. J Environ Biol 2013;34:729-32.
4. Casella TM, Eparvier V, Mandavid H, Bendelac A, Odonne G, Dayan L, Duplais C, Espindola LS, Stien D. Antimicrobial and cytotoxic secondary metabolites from tropical leaf endophytes: isolation of antibacterial agent pyrrocidine C from *Lewia infectoria* SNB-GTC2402. Phytochemistry 2013; 96:370-7.
5. Quarles W. New biopesticides for IPM and organic production. IPM Pract 2013;33:1-20.
6. Klaiklay S, Rukachaisirikul V, Phongpaichit S, Pakawatchai C, Saithong S, Buatong J, Preedanon S, Sakayaroj J. Anthraquinone derivatives from the mangrove-derived fungus *Phomopsis* sp. PSU-MA214. Phytochem Lett 2012;5:738-42.
7. Rukachaisirikul V, Rodglin A, Phongpaichit S, Buatong J, Sakayaroj J. α-Pyrone and seiricuprolide derivatives from the mangrove-derived fungi *Pestalotiopsis* spp. PSU-MA92 and PSU-MA119. Phytochem Lett 2012;5:13-7.
8. Nambiar GR, Raveendran K. Manglicolous marine fungi on *Avicennia* and *Rhizophora* along Kerala Coast (India). Middle East J Sci Res 2009;4:48-51.
9. Tarman K, Lindequist U, Wende K, Porzel A, Arnold N, Wessjohann LA. Isolation of a new natural product and cytotoxic and antimicrobial activities of extracts from fungi of Indonesian marine habitats. Mar Drugs 2011;9:294-306.
10. Sumampouw M, Bara R, Awaloei H, Posangi J. Uji efek antibakteri jamur endofit akar bakau *Rhizophora stylosa* terhadap bakteri *Staphylococcus aureus* dan *Escherichia coli*. J e-Biomedik 2014:2.
11. Kartika R, Bodhi W, Kepel B, Bara R. Uji daya hambat jamur endofit akar bakau *Rhizophora apiculata* terhadap bakteri *Staphylococcus aureus* dan *Escherichiae coli*. J e-Biomedik 2014:2.
12. Li H, Huang H, Shao C, Huang H, Jiang J, Zhu X, Liu Y, Liu L, Lu Y, Li M, et al. Cytotoxic norsesquiterpene peroxides from the endophytic fungus *Talaromyces flavus* isolated from the mangrove plant *Sonneratia apetala*. J Nat Prod 2011;74: 1230-5.
13. Phoanda TC, Bara R, Wowor PM, Posangi J. Uji efek antibakteri jamur endofit akar tumbuhan bakau (*Bruguiera gymnorrhiza*) terhadap bakteri *Escherichia coli* dan *Staphylococcus aureus*. J e-Biomedik 2014:2.
14. Bhimba BV, Agnel Defora Franco DA, Jose GM, Mathew JM, Joel EL. Characterization of cytotoxic compound from mangrove derived fungi *Irpex hydnoides* VB4. Asian Pac J Trop Biomed 2011;1:223-6.
15. Chaeprasert S, Piapukiew J, Whalley AJ, Sihanonth P. Endophytic fungi from mangrove plant species of Thailand: their antimicrobial and anticancer potentials. Bot Mar 2010; 53:555-64.
16. Swe A, Jeewon R, Pointing SB, Hyde KD. Taxonomy and

molecular phylogeny of *Arthrobotrys mangrovispora*, a new marine nematode-trapping fungal species. Bot Mar 2008;51: 331-8.
17. Chen G, Lin Y, Vrijmoed LL, Fong WF. A new isochroman from the marine endophytic fungus 1893. Chem Nat Compd 2006;42:138-41.
18. Alongi DM. Present state and future of the world's mangrove forests. Environ Conserv 2002;29:331-49.
19. FAO Regional Forestry Commissions. The state of forest resource: a regional analysis [Internet]. Rome: Food and Agriculture Organization; 2010 [cited 2015 Feb 20]. Available from: http://www.fao.org/docrep/013/i2000e/i2000e01.pdf.
20. Höller U. Isolation, biological activity and secondary metabolite investigations of marine-derived fungi and selected host sponges [dissertation]. Braunschweig: Universität Carolo-Wilhelmina; 1999.
21. Harwig J, Scott PM. Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. Appl Microbiol 1971; 21:1011-6.
22. Ananda K, Sridhar KR. Diversity of endophytic fungi in the roots of mangrove species on the west coast of India. Can J Microbiol 2002;48:871-8.
23. Schulz B, Wanke U, Draeger S, Aust HJ. Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. Mycol Res 1993;97:1447-50.
24. Poyton RO. The isolation and occurrence of *Hyalochlorella marina*. J Gen Microbiol 1970;62:189-94.
25. Torzilli AP, Sikaroodi M, Chalkley D, Gillevet PM. A comparison of fungal communities from four salt marsh plants using automated ribosomal intergenic spacer analysis (ARISA). Mycologia 2006;98:690-8.
26. Polishook JD, Bills GF, Lodge DJ. Microfungi from decaying leaves of two rain forest trees in Puerto Rico. J Ind Microbiol 1996;17:284-94.
27. Meyling NV. Methods for isolation of entomopathogenic fungi from the soil environment. Frederiksberg: University of Copenhagen; 2007.
28. Burtseva YV, Verigina NS, Sova VV, Pivkin MV, Zvyagintseva TN. O-glycosylhydrolases of marine filamentous fungi: β-1,3-glucanases of *Trichoderma aureviride.* Appl Biochem Microbiol 2003;39:475-81.
29. Kang SC, Park S, Lee DG. Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae.* J Invertebr Pathol 1999;73:276-81.
30. Delalibera I Jr, Hajek AE, Humber RA. Use of cell culture media for cultivation of the mite pathogenic fungi *Neozygites tanajoae* and *Neozygites floridana*. J Invertebr Pathol 2003;84: 119-27.
31. Osono T. Colonization and succession of fungi during decomposition of *Swida controversa* leaf litter. Mycologia 2005;97:589-97.
32. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. New York: Academic Press; 1990. p. 315-22.
33. Michaelsen A, Pinzari F, Ripka K, Lubitz W, Piñar G. Application of molecular techniques for identification of fungal communities colonizing paper material. Int Biodeterior Biodegrad 2006;58:133-41.
34. Brock PM, Döring H, Bidartondo MI. How to know unknown fungi: the role of a herbarium. New Phytol 2009;181:719-24.
35. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997;25:4876-82.
36. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406-25.
37. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 1985;39:783-91.
38. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980;16:111-20.
39. Supriyono A. Biologische aktive naturstoffe aus tropischen schämmen Südostasiens [dissertation]. Würzburg: Bayerischen Julius-Maximilians-Universität; 1997.
40. Ladhari A, Laarif A, Omezzine F, Haouala R. Effect of the extracts of the spiderflower, *Cleome arabica*, on feeding and survival of larvae of the cotton leafworm, *Spodoptera littoralis*. J Insect Sci 2013;13:61.
41. Ellman GL, Courtney KD, Andres V Jr, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.
42. Paz Z, Bilkis I, Gerson U, Kerem Z, Sztejnberg A. Argovin, a novel natural product secreted by the fungus *Meira argovae*, is antagonistic to mites. Entomol Exp Appl 2011;140:247-53.
43. Abbott WS. A method of computing the effectiveness of an insecticide. J Econ Entomol 1925;18:265-7.
44. Finney DJ. Probit analysis. 3rd ed. London: Cambridge University Press; 1971.
45. Gibbons JG, Rokas A. The function and evolution of the *Aspergillus* genome. Trends Microbiol 2013;21:14-22.
46. An CY, Li XM, Luo H, Li CS, Wang MH, Xu GM, Wang BG. 4-Phenyl-3,4-dihydroquinolone derivatives from *Aspergillus nidulans* MA-143, an endophytic fungus isolated from the mangrove plant *Rhizophora stylosa.* J Nat Prod 2013;76:1896-901.
47. Bhimba BV, Agnel Defora Franco DA, Mathew JM, Jose GM, Joel EL, Thangaraj M. Anticancer and antimicrobial activity of mangrove derived fungi *Hypocrea lixii* VB1. Chin J Nat Med 2012;10:77-80.
48. Joel EL, Bhimba BV. Evaluation of secondary metabolites from mangrove associated fungi *Meyerozyma guilliermondii*. Alex J Med 2013;49:189-94.
49. Elavarasi A, Rathna GS, Kalaiselvam M. Taxol producing mangrove endophytic fungi *Fusarium oxysporum* from *Rhizophora annamalayana*. Asian Pac J Trop Biomed 2012;2: S1081-5.
50. Bai ZQ, Lin X, Wang Y, Wang J, Zhou X, Yang B, Liu J, Yang X, Wang Y, Liu Y. New phenyl derivatives from endophytic fungus *Aspergillus flavipes* AIL8 derived of mangrove plant *Acanthus ilicifolius*. Fitoterapia 2014;95:194-202.
51. Chen Y, Mao W , Tao H, Zhu W, Qi X, Chen Y, Li H, Zhao C, Yang Y, Hou Y, et al. Structural characterization and antioxidant properties of an exopolysaccharide produced by the mangrove endophytic fungus *Aspergillus* sp. Y16. Bioresour Technol 2011;102:8179-84.

52. Liu D, Li XM, Meng L, Li CS, Gao SS, Shang Z, Proksch P, Huang CG, Wang BG. Nigerapyrones A-H, α-pyrone derivatives from the marine mangrove-derived endophytic fungus *Aspergillus niger* MA-132. J Nat Prod 2011;74:1787-91.
53. Naikwade P, Mogle U, Sankpal S. Phyloplane mycoflora associated with mangrove plant *Ceriops tagal* (Perr.). Sci Res Rep 2012;2:85-7.
54. Qiao MF, Ji NY, Liu XH, Li K, Zhu QM, Xue QZ. Indoloditerpenes from an algicolous isolate of *Aspergillus oryzae*. Bioorg Med Chem Lett 2010;20:5677-80.
55. Miao FP, Li XD, Liu XH, Cichewicz RH, Ji NY. Secondary metabolites from an algicolous *Aspergillus versicolor* strain. Mar Drugs 2012;10:131-9.
56. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: a convinient general bioassay for active plant constituents. Planta Med 1982;45:31-4.
57. Busi S, Peddikotla P, Upadyayula SM, Yenamandra V. Isolation and biological evaluation of two bioactive metabolites from *Aspergillus gorakhpurensis*. Rec Nat Prod 2009;3:161-4.
58. Busi S, Rajkumari J, Hnamte S. Feeding deterrence, acute toxicity and sublethal growth effects of kojic acid isolated from *Aspergillus funiculosus*. Nat Prod J 2014;4:18-22.
59. Hummelbrunner LA, Isman MB. Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (Lep., Noctuidae). J Agric Food Chem 2001;49:715-20.