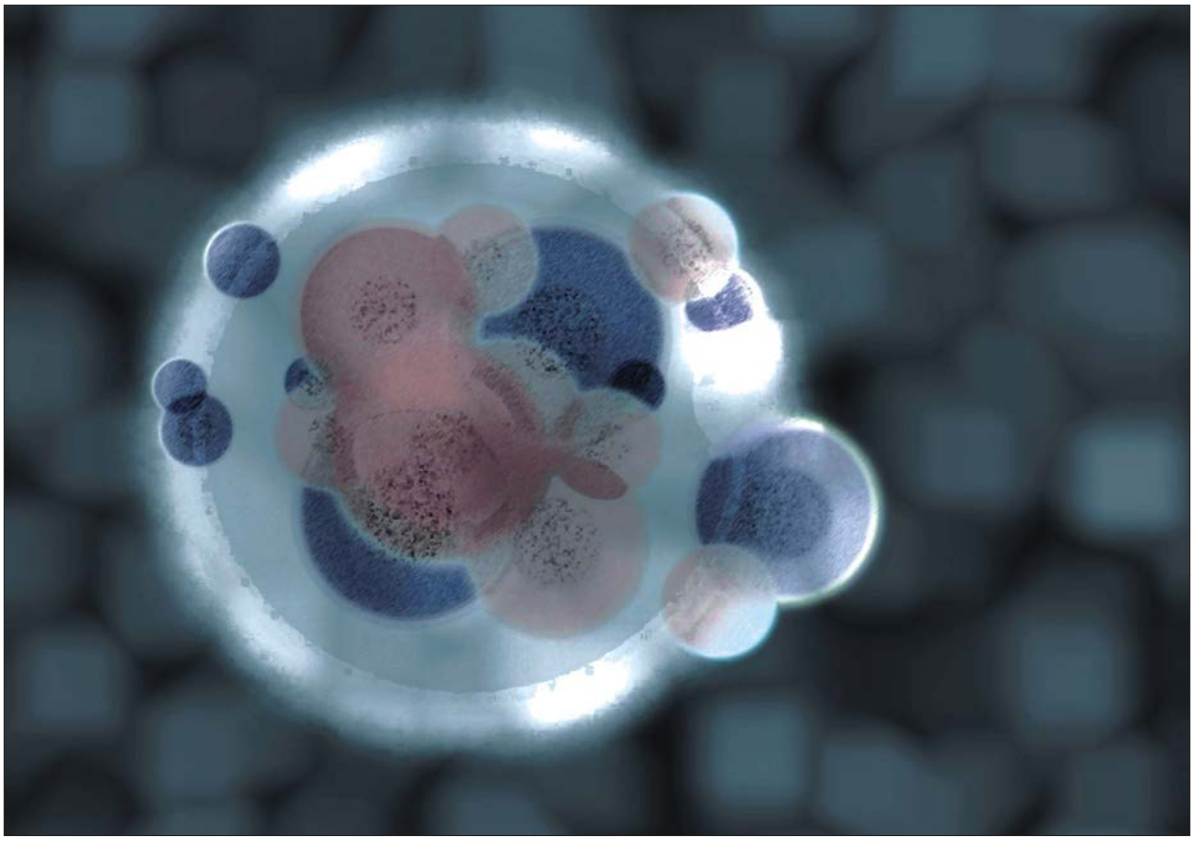

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Research Article
Bioactivity of Fungi *Trichoderma reesei* Associated with Sponges *Stylissa flabelliformis* Collected from National Park West Bali, Indonesia

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Abstract

Objective: In this study, antimicrobial and cytotoxic properties of ethyl acetate extract of fermented *Trichoderma reesei* (*T. reesei*)fungi associated with the sponge *Stylissa flabelliformis* (*S. flabelliformis*) was tested. **Materials and Methods:** Fermentation of *T. reesei* was carried out in the saboroud dextrosa saline broth. The fermented filtrate was extracted using ethyl acetate. The ethyl acetate extract was tested for its antimicrobial and cytotoxic activity. Thin-layer chromatography and gas chromatography mass spectrometry were performed to determine the metabolite classes of compounds responsible for antimicrobial and cytotoxic activity. **Results:** Ethyl acetate extract of *T. reesei* was active against the bacteria *Staphylococcus aureus* ATCC 29213, *Eschericia coli* ATCC 25922 and *Candida albicans* ATCC 10231. The ethyl acetate extract was also active against cancer cells T47D and Raji cells, with IC_{50} values of 270 and 470 μg mL^{-1}, respectively. **Conclusion:** An ethyl acetate extract of *Trichoderma reesei* fermentations showed antimicrobial and cytotoxic activity. The compounds contained in the extract of *T. reesei* are terpenoids or phenyl propane derivatives.

Key words: Fungi-associated sponge, *Trichoderma reesei*, *Stylissa flabelliformis*, cytotoxic test, antimicrobial test

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

For many organisms, especially sponges, the biological nature of microbes associations has never been rigorously investigated or defined; these associations may involve symbiotic, specific and permanently associated but not symbiotic or merely commensally present microorganisms. In terms of their secondary metabolism, these life forms are enormously productive and evoke fascinating ecological questions[1]. Their investigation is, however, equally important due to the potential of marine natural products as drugs.

Sponges are sessile, ancient metazoans from the phylum Porifera. A large portion of the sponge mass, 35-60%, may be composed of microorganisms, including cyanobacteria, heterotrophic bacteria, unicellular algae and fungi[2]. Thus, it is possible that a compound reportedly produced by a sponge is actually produced by its symbiotic microbes[1]. For example, Microluside A isolated from the marine sponge *Spheciospongia vagabunda* is known to be produced by *Micrococcus* sp. EG45, a bacterium associated with sponges[3]. The case of bryostatins already showed that metabolites initially assigned to a host organism are of microbial origin[4-6]. In several cases, there is evidence from experiments that sponge-derived fungi are the true biosynthetic sources of sponge-derived secondary metabolites such as jasplakinolide[7].

Natural marine products also have issues in terms of the availability of raw materials. The wild harvest of many marine organisms is banned because it can threaten population sustainability. Alternatively, the use of microorganisms is considered more profitable. It is known that many isolated symbiotic microorganisms from natural marine products produce the same compounds as their hosts[8]. One such microorganism is a fungus. Fungi grow faster than their sponge hosts and can be produced on a large scale by fermentation[9].

Previous studies[10] showed that the sponge *Stylissa flabelliformis* hosts at least 10 fungi that have antimicrobial activity against all tested microbes. This paper describes fermentation, microbial testing and cytotoxic testing of an ethyl acetate extract from the fungus *T. reesei* associated with the sponge *S. flabelliformis* and attempt to identify the potential derivative compounds.

MATERIALS AND METHODS

All chemicals used in this research were of analytical grade. The study was carried out between April, 2016 and May, 2017.

Materials: *Stylissa flabelliformis* sponge and *Trichoderma reesei* fungus are kept at the Department of Pharmaceutical Biology, Faculty of Pharmacy, UGM, Yogyakarta, Indonesia. Other materials include the following: Saboroud dextrose (Broth and Agar, Oxoid), ethyl acetate (Merck), paper disc (diameter 6 mm, no. 2668; Schleicher and Schu¨ll, Germany), standard antibiotic disc (chloramphenicol (20 µg/disc) and kanamycin (20 µg/disc, Sigma), methanol (Merck), Roswell Park Memorial Institute 1640 (RPMI 1640; Sigma) media, Dulbecco's Modified Eagle's Medium (DMEM; Sigma), phosphate-buffered saline (PBS) 10% v/v (Gibco), Fungison 0.5%, penicillin streptomycin 1% v/v (Gibco) and 3-(4,5-dimetiltiazol-2-il)-2-5-difenil tetrazoliumbromida (MTT; Sigma).

Equipment: The equipment use for this study include an incubator (heracell (heraeus) Kendro Laboratory Products, Germany), Gas Chromatography Mass Spectrometry (GC-MS; Shimadzu GCMS-QP2010SE), a laminar air flow (Labconco purifierTM class II) inverted microscope (Axiovert 25) and a haemocytometer (Nebauer).

Cultivation and extraction of fungal secondary metabolite: The culture of *T. reesei* was performed by scraping the fungal spores on the surface of saboroud dextrose agar plates and incubating them at room temperature for 7 days. For the production of secondary metabolites, the fungi were cultivated at 25°C for 11 days on saboroud dextrose saline broth (Sabouraud Broth 40 g, seawater 1000 mL) on a rotary shaker (120 rpm) at room temperature (25°C). For initial analyses of the natural products, the cultures were lyophilized and extracted with ethyl acetate; the extractions were then evaporated in a rotary evaporation at 60°C until dry.

Antimicrobial activity: Antimicrobial activity was assessed using the diffusion method described by Badji *et al.*[11] and Oskay *et al.*[12]. The ethyl acetate extract, with a concentration of 10 mg mL^{-1}, was dissolved in an organic solvent. A total of 10 µL of the extract was poured onto a paper disc. Standard antibiotic discs of chloramphenicol and kanamycin were used as the positive control and discs saturated with 10% methanol were used as the negative control. After all of the solvent had evaporated, each paper disc was placed on the surface of a medium that already contained the test microbes. These test plates were incubated at 37°C for 24 h, after which the diameter of resistance was measured.

Cytotoxic evaluation by MTT assay: The cytotoxic evaluation was performed following an established standard procedure[13].

Fungal cells were distributed into 96-well plates at a concentration of 5×10^3 cells/well and incubated at room temperature. Twenty-four hours later, the medium was discarded and the cells were washed with PBS. Various test compounds were added to the wells either singly or in combination. Subsequently, the cells were incubated again for 24 h in a 5% CO_2 incubator at 37°C. After 24 h of incubation, the medium was discarded and the cells were washed with 100 µL PBS. Each well then received 100 µL of 0.5 mg mL^{-1} MTT reagent in DMEM medium and was incubated for 3-4 h in a 5% CO_2 incubator at 37°C. Live cells reacted with MTT to form a purple colour. The MTT reaction was stopped with a stopper reagent (10% SDS in 0.1 N HCl), followed by an overnight incubation at room temperature in the dark (covered with aluminium foil). The following day, the plate was shaken for 10 min and the absorbance was read with an ELISA reader at a wavelength of 595 nm.

GC-MS (Shimadzu GCMS-QP2010SE) analysis: GC-MS was performed on a Rtx-5MS capillary column (30×0.25 mm, I.d.) coated with a 0.25 µm film of 5% phenyl methyl siloxane used for separation. The injection temperature was set to 200°C. The column was held at 70°C for 5 min and then the temperature was raised to 300°C at a constant rate of 10°C min^{-1}. Once the temperature reached 300°C, the column was held at constant temperature for 5 min. The ion source temperature was 200°C and the interface temperature was 250°C with a splitless injection volume of 1 µL. The data from GC-MS were compared with the WILEY7.LIB library.

RESULTS AND DISCUSSION

Isolation of fungi from *Stylissa flabelliformis* sponge generated cultures of at least 10 species of fungi. All 10 of these fungi were tested against microbes such as *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231. *Trichoderma reesei* associated with the sponge *S. flabelliformis*, obtained from National Park West Bali (Indonesia), is one of the fungi active against some microbes. The macroscopic profile (growth in the saboroud saline medium) and microscopic profile of this species are illustrated in (Fig.1a and b). Molecular identification also confirmed that the species shown in Fig. 1 was *T. reesei*.

The spores (conidia) typically appear dry but in some species, they may be held in drops of clear green or yellow liquid. Conidia of most species are ellipsoidal, 3-5×2-4 µm (L/W≥1.3); globose conidia (L/W<1.3) are rare. Conidia are typically smooth but tuberculate to finely warty conidia are known in a few species[14].

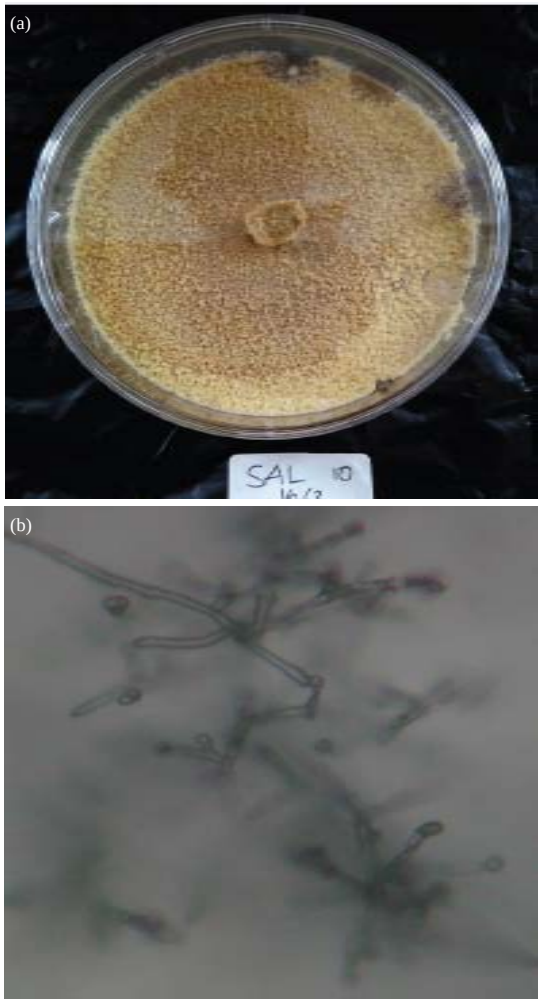

Fig. 1(a-b): Macroscopic and microscopic morphology of fungus *T. reesei* (in the saboroud saline medium), (a) *T. reesei* (growth on day 7) and (b) *T. reesei* (under microscope view; scale 1:200)

Antimicrobial evaluations of ethyl acetate extract fermentation of fungi *Trichoderma reesei:* Fermentation of *T. reesei* was carried out for 14 days on a rotary shaker at 120 rpm using a medium of saboroud broth and seawater. The results of the antimicrobial test against the fermented ethyl acetate extract at concentration 1000 µg mL^{-1} are indicated in Fig. 2. The ethyl acetate extract began to show antimicrobial properties on day 2 of the fermentation process, as shown in Fig. 3. The antimicrobial activity of the ethyl acetate extract reached its peak on day 11 of the fermentation. This observation suggests that the secondary metabolites, which have the greatest antimicrobial activity, were generated on day 11. The comparison of day-to-day results of the antimicrobial activity test is shown in Fig. 3.

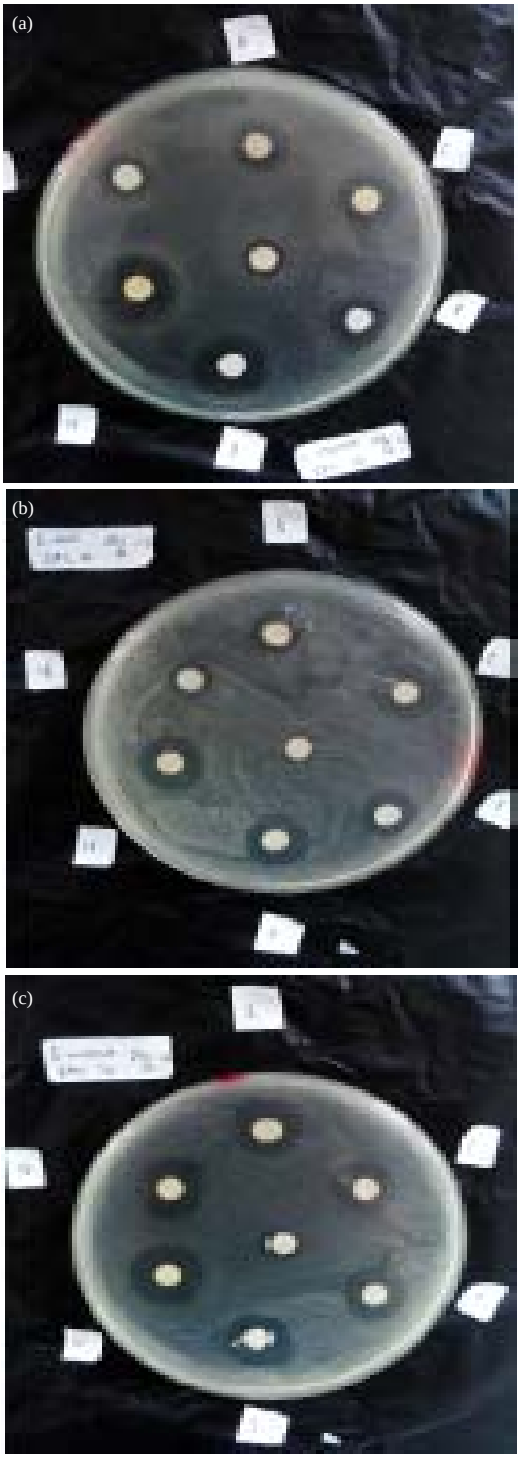

Fig. 2(a-c): Antimicrobial effects of fermented fungus (*T. reesei*), (a) *C. albicans*, (b) *E. coli* and (c) *S. aureus*

Cytotoxic evaluations of ethyl acetate extract from the fermentation of *Trichoderma reesei*: The cytotoxic test was performed on two cancer cell cultures: Raji cells and T47D cells. Raji cells are derived from the culture of a lymphoblastic cell line derived from Burkitt lymphoma. Meanwhile, T47D cells are a continuous cell line isolated from breast ductal tumour tissue of a woman aged 54 years. Continuous cell lines commonly used in cancer research *in vitro* can be handled easily, have unlimited replication capabilities, are highly homogeneous and can easily be replaced with frozen stock in case of contamination[15]. T47D cells are a particular kind of epithelial cell morphology. The cytotoxic test was executed with a cell density of 1.5×104.

Table 1: Metabolite compounds of *T. reesei* associated with sponge *S. flabelliformis* by GC-MS Shimadzu type QP2010SE

Name of compound	Concentration (%)
1,2-Ethanediol, 1-phenyl- (CAS) Styrene glycol	24.70
Alpha -bisabololoxide-B	4.17
Veridifloror	13.15
1-Octadecene (CAS).alpha.-Octadecene	1.42
1,4-Diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	17.04
2,5,5-Trimethyl-3,6-heptadien-2-ol	3.12
1,4-Diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	11.79
Methyl-1,4-Dihydroxy-2-naphthoate	3.14
1,4-Diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	19.31
1,4-Diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	2.16

The results of the study are shown in Fig. 4, which shows the occurrence of damage to Raji cells and T47D cells with increasing dosage of the fungal extract. The IC_{50} value for the Raji cells against the ethyl acetate was 470 μg mL^{-1}, while the IC_{50} for T47D cells was 270 μg mL^{-1}.

***Trichoderma reesei* ethyl acetate extract:** Thin layer chromatography (TLC) was performed to determine the classes of compounds contained in the ethyl acetate extract of fermented *T. reesei*. A chromatogram from this TLC analysis is shown in Fig. 5. Phytochemical detection was carried out by developing the ethyl acetate extract in a mobile phase solvent containing hexane and ethyl acetate (3:1 v/v) with the addition of 1 drop of glacial acetic acid. The ethyl acetate extract showed a positive reaction only on citroborate and anisaldehyde reagents, as indicated in Fig. 5. Thus, the ethyl acetate extract may contain terpenoids or phenyl propane derivatives.

Class of compounds identification of ethyl acetate extract by Gas Chromatography Mass Spectrometry (GC-MS): Identification of the compounds contained in the ethyl acetate extracts based on molecular weight can be done with GC-MS. The peak data were then cross-checked with a library (WILEY7.LIB). The potential compounds according to the GC-MS analysis are presented in Table 1.

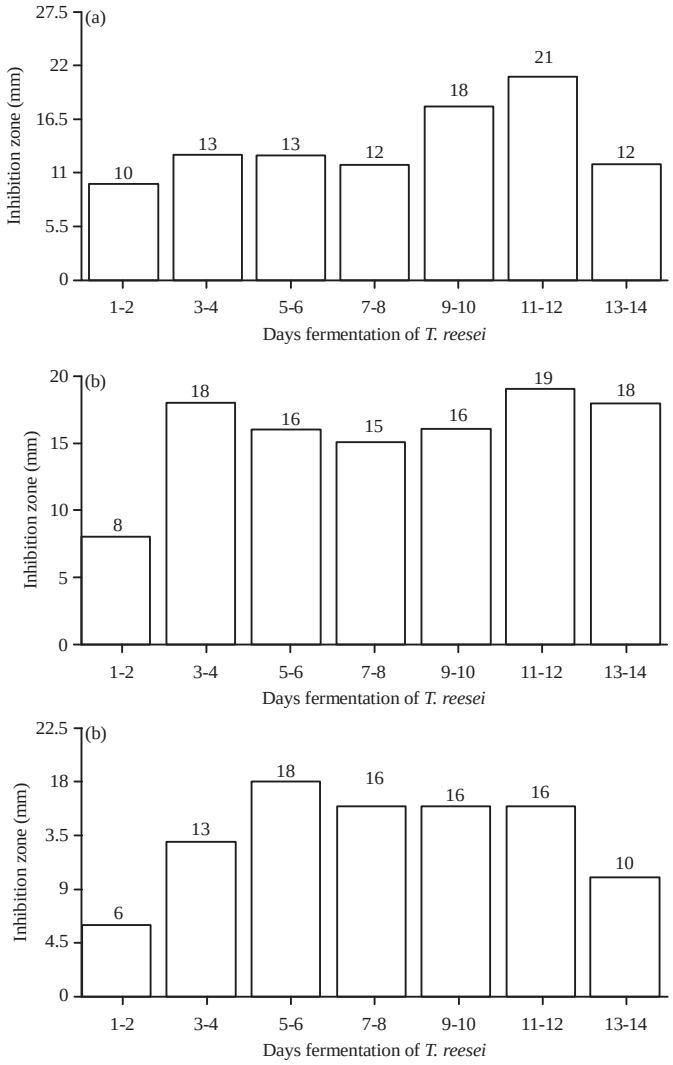

Fig. 3(a-c): Fermentation days of *Trichoderma reesei* vs inhibition zone of microbes (a) *T. reesei* vs *Candida albicans*, (b) *T. reesei* vs *S. aureus* and (c) *T. reesei* vs *E. coli*

Cyclohexanone was one of the metabolite compounds identified in extracts from *T. reesei* associated with *S. flabelliformis*. Cyclohexanone was previously isolated from fungus *Pestalotiopsis fici*, which showed antimicrobial activity against *A. fumigatus*[16]. In addition to cyclohexanone, phenol compounds were also metabolites with potential antimicrobial features.

In this study, found antimicrobial and cytotoxic activity of *Trichoderma reesei* fungi that associate with the sponge *Stylissa flabelliformis*. It is also found that the major compounds in the ethyl acetate extract of this fungus belong to the terpenoid group. *Stylissa flabelliformis* is a sponge of division *Demospongiae*[17] that has been known to produce jaspamide/jasplakinolide[9]. This compound has cytotoxic and antimicrobial activity[9]. Research on the isolation of microbes associated with sponges has been widely practiced. Although most sponge metabolites are believed to be produced by the sponge itself because the sponge contains symbiotic microorganisms, it is necessary to examine where the sponge metabolites actually originate. Often, metabolite compounds have structures similar to those of metabolites produced by sponges, which are taxonomically distinct[18]. This finding suggests that these compounds may originate in sponge-associated microorganisms and are responsible for the production of various bioactive compounds. Thus, it is estimated that some sponge metabolites are produced by symbiotic microorganisms.

Symbiotic microorganisms in the sponge can be a source of natural products and recent studies have shown that the metabolites produced by sponges are synthesized by their

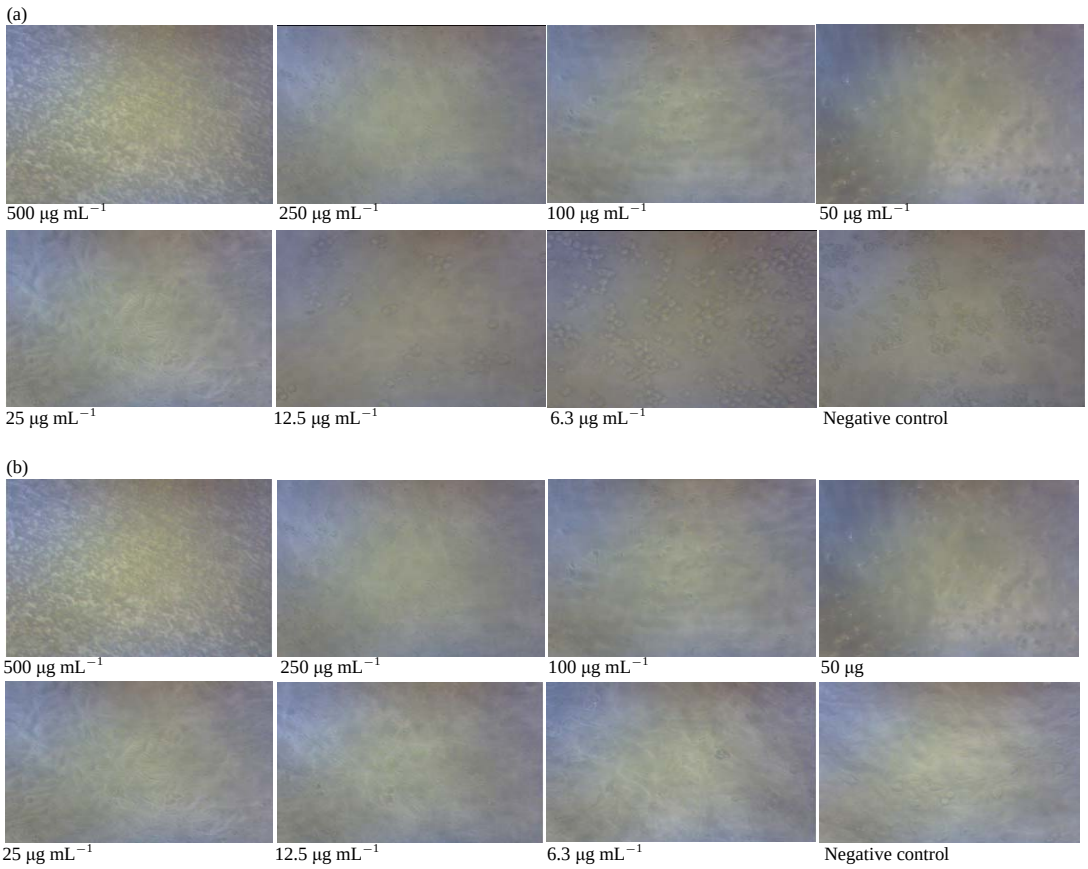

Fig. 4(a-b): Cancer cell profile (a) Raji cells and (b) T47D cells after treatment with *Trichoderma reesei* ethyl acetate extract

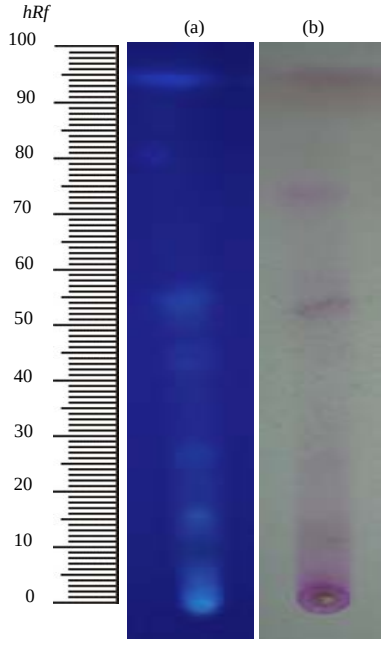

Fig. 5(a-b): Chromatogram of ethyl acetate extract from *T. reesei*, (a) Detection with Citroborate (UV366) and (b) Detection with Anisaldehyde

symbiotic microorganisms. If the symbiotic microorganisms of the metabolite-producing sponge can be obtained and cultured, then the mass-production of certain metabolites is possible[1,3].

Reports on new natural products produced by symbiotic fungi (Ascomycota) have recently increased. HIV-1 Sorbicillactone A is found in *Penicillium chrysogenum* associated with the sponge *Ircinia fasciculata*[19]. H1N1 Pyronepolyene C-glucoside iso-D8642-2-6 compounds are detected in *Epicoccum* sp. JIY40 isolated from the sponge Callyspongia sp[20]. H3N2 (Z)-5(Hydroxymethyl)-2-(60)-methylhept-20-en-20yl)-phenol are found in *Aspergillus sydowii* ZSDSI-F6 isolated from an unidentified sponge[21]. Antimicrobial activities are detected in *Streptomyces* sp. isolated from the sponge *Aplysina fistularis*[22].

CONCLUSION

An ethyl acetate extract of *Trichoderma reesei* fermentations showed antimicrobial and cytotoxic activity. Most of the compounds contained in the ethyl acetate extract of *T. reesei* were terpenoids.

SIGNIFICANCE STATEMENT

This study was conducted to find microbes that associate with the sponge *S. flabelliformis* in an effort to produce compounds that have cytotoxic and antimicrobial activity.

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