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Antiproliferative and Antimicrobial Activities of Endophytic Fungus Isolated from *Erycibe Elliptilimba*

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ABSTRACT

Background: *Erycibe elliptilimba* Merr.&Chun. is a Thai herb in the family Convolvulaceae that has long been used in traditional medicine to treat various diseases including infections and malignancies. Its methanolic extract has antiproliferative activities against various human cancer cell lines with potential ED50 values.

Objective: The antiproliferative activities exhibited from this plant should be investigated to ensure that the effects were from the plant itself. Therefore, the endophytic fungus from this special plant was isolated and evaluated for their antiproliferative and antimicrobial activities.

Methods: The isolation of fungal endophytes from the stems of *Erycibe elliptilimba* Merr.&Chun. was identified by macroscopic and microscopic morphology. Fungal extraction was demonstrated using thin layer chromatography of the did broth extract. Four human breast cancer cell lines, two human lung eancer cell lines, and one human colon cancer cell line were used to evaluate the antiproliferative activity of the crude broth extract on the cell growth and determine of antibacterial activities.

Results: The results revealed that only one species, *Trichoderma ovalisporum*, was isolated and its cultured broth extract did not provide an antiproliferative effect against any human cancer cell line nor antimicrobial activities.

Conclusion: Based on this study, the antiproliferative activities against several human cancer cell lines of *Erycibe elliptilimba*, Merr.&Chun. extract, in recent reports may depend upon the active compounds in the plant itself.

Keywords: *Trichoderma ovalisporum*, antiproliferative activities, antimicrobial activities, endophytic fungus, *Erycibe elliptilimba* Merr.&Chun.

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Erycibe elliptilimba Merr.&Chun., one of the Thai herbs in the Convolvulaceae family, has long been used in Thai traditional medicine for treating various diseases. From Thai Pharmacopoea, the stems of this plant which are commonly used as one of the ingredients in Thai herbal remedies has been prescribed as an antifebrile from unknown fever including infections and malignancies.[1,2] From the previous reports, the potential antiproliferative properties of the methanolic extract from this plant against several human cancer cell lines has been demonstrated.[3,4] This remarkable information confirms the usage of this plant as a natural anticancer agent. However, healthy plants usually have the endophytic fungi that colonize the stems which may also be the sources of some biological activities especially anticancer and antimicrobial properties. Thus, the antiproliferative activities of this plant which was previously demonstrated, perhaps, may be influenced by the metabolites from its endophytic fungus. Therefore, this prompted us to evaluate the antiproliferative and antimicrobial effects of endophytic fungus isolated from this medicinal plant.

MATERIALS AND METHODS

1. Collection of plant samples

The stems of *Erycibe elliptilimba* Merr.&Chun. were collected from the forest area at Ratchaburi province, in the eastern part of Thailand, and were identified by Miss Leena Phuphathanapong, a taxonomist at the Forest Herbarium (BKF), Royal Forest Department,

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Ministry of Agriculture and Cooperatives, Bangkok, Thailand. A voucher specimen No. 015461 was deposited at the same place.

2. Isolation of fungal endophytes

Surfaces of stem samples were cleaned in tap water and air-dried. Then, they were sterilized by soaking in 70% ethanol for 1 minute and in sterile water for 5 minutes three times. The inner bark segments of stems were cut into small pieces 0.5 x 1 cm. using a sterile blade and cleaned in sterile water for 1 minute three times. They were deposited onto a potato dextrose agar (PDA) plate and incubated at room temperature for 7 days. The fungal isolate was identified by macroscopic and microscopic morphology.

3. Fungal extraction

The fungal isolate was subcultured on a PDA plate and incubated at 25°C for 7 days. Ten pieces, 1x1 cm^2, of the agar with growing fungus were put into a 1 litre Erlenmeyer flask containing 200 ml of malt extracted broth (MEB) or yeast extract sucrose broth. After incubation at room temperature for 21 days under stationary conditions, the fungal culture was filtered to separate the mycelium portion. The filtered broth was then extracted with 200 ml of dichloromethane three times. The organic phase was evaporated air-dried and weighed until a constant weight was obtained to get the crude broth extract.[5] Thin layer chromatography (TLC) of the crude broth extract was demonstrated in Fig 1.

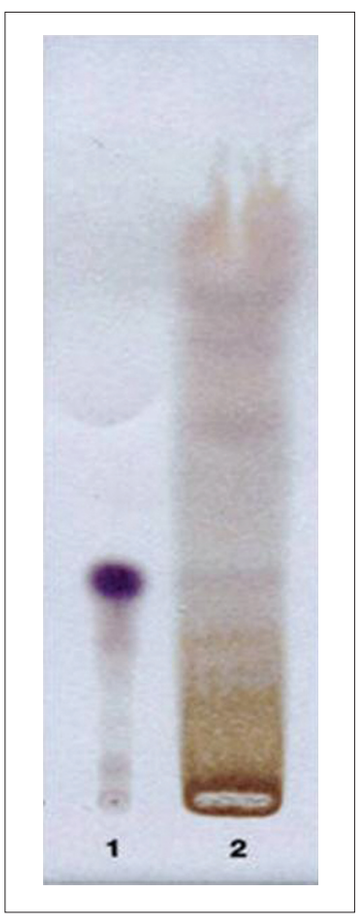


Fig 1. TLC pattern of the extracts from crude broth extract. TLC analysis was determined using Chloroform:Methanol = 85:15 as a mobile phase and sprayed by 10% sulfuric acid. Track 1: Charantin (Phytosteryl glucoside); Track 2: Alcohol extract of MEB.

4. Preparation of fungal extract

For the antimicrobial experiment, crude broth extract was dissolved in dimethylsulphoxide (DMSO, Sigma, USA) to obtain a stock concentration of 10,000 µg/ml. For the antiproliferative experiment, the extract was dissolved in 100% DMSO to be stock solution. The final concentrations at 1, 10, 100, 500 µg/ml were achieved by diluting the stock with the medium.

5. Cell lines and culture

Four human breast cancer cell lines, SKBR3, MCF7, T47D and MDA-MB435, were kindly provided by Dr. Pornchai O-charoenrat (Mahidol University, Bangkok). T47D and MCF7 are breast cancer cell lines that are ER receptor positive and HER2/*neu* negative, whereas SKBR3 breast cancer cells have over-expression of HER2/*neu* receptor, absence of ER receptor. MDA-MB435 is absent from both ER receptor and HER2/*neu* expression.[6] Two human lung cancer cell lines, A549 and SK-LU1, were purchased from ATCC (American Type Culture Collection). A549 is a cisplatin resistant adenocarcinoma lung cancer cell whereas SK-LU1 is adenocarcinoma lung cancer cell which is sensitive to cisplatin. Caco-2, kindly provided by the Department of Medical Sciences, Ministry of Public Health, is a human colon cancer cell which expresses the MDR receptor. All cell lines except SK-LU1 were cultured in DMEM/F12 (Gibco, Thailand) whereas SK-LU1 was cultured in MEM (Gibco, Thailand) supplemented with non essen-tial amino acid and 1 mM sodium pyruvate. All media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Thailand) and 1% penicillin and streptomycin (Gibco, Thailand). The cells were incu-bated at 37°C in a humidified atmosphere with 5% CO_2 and subcultured with 0.05% trypsin-EDTA (Gibco, Thailand).

6. Determination of antiproliferative activity

To evaluate the antiproliferative activity of crude broth extract on the cell growth, cells at $1x10^4$ cells/well were seeded in 96 well plates containing 100 µl of the culture medium. After 24-hour incubation, cells were treated with the extract at the concentration of 1, 10, 100, and 500 µg/ml for 48 hours. Then MTT (3-(4, 5-dimethyl thizol-2-yl)-2,5-diphenyl tetrazolium bromide) 50 µl (1 mg/ml in PBS) were added in each well for 4 hours. The MTT solution was removed and 100 µl of DMSO were added.[7,8] The experiments were performed in triplicate with three independent experiments. Absorbance at 595 nm. of the dissolved solutions was detected by a microplate ELISA reader (Biorad, USA).[9] Data were calculated as percentage viability using the following formula:

Cell death (%) = [(control O.D. - sample O.D.)/control O.D.] x 100

Cell viability (%) = 100 - (% cell death)

7. Determination of antibacterial activities

7.1. Anti-Candida activity testing

Minimum inhibitory concentration (MIC) of the extract against *Candida* species was determined based on a broth microdilution method with some modifica-

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tions.[10] *Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019 were incubated at 37°C on Sabouraud dextrose agar for 24 hours. At least 10 colonies were suspended in 5 ml of 0.85% NaCl to a density of 0.5 McFarland that was calibrated with a spectrophotometer at 530 nm. The suspension was diluted at 1:1000 in 10 ml of MOPS buffered RPMI 1640 with L-glutamine. The suspension of 100 µl was dispensed into each well of 96-well plates. The crude broth extract were serially two-fold diluted in a concentration range from 4 to 2048 µg/ml in 10 ml sterile test tubes containing test broth. Then, 100 µl from the serial dilutions was transferred into 3 consecutive wells of the 96-well plates. They were incubated at 37°C for 48 hours. Growth control well and sterility control well consisted of organism only and medium only, respectively. Amphotericin B was used as a positive control. The MIC was defined as the lowest concentration of the extract that inhibited growth of the organisms.

7.2. Anti-*Escherichia coli* and *Staphylococcus aureus* testing

Escherichia coli ATCC 25923 and *Staphylococcus aureus* ATCC 25922 were used in this study. The antimicrobial test was carried out by a disc diffusion method using a 0.5 McFarland standard inoculum spread on Mueller-Hinton agar surface. The crude broth extract was diluted to a final concentration of 10,000, 5,000, and 2,500 µg/ml in test broth. Ten microlitres of each solution was impregnated into sterile 6 mm diameter filter discs. Plates were allowed to dry before the discs were applied, and then incubated overnight (16-18 hours) prior to the measurement of zone diameters. Negative controls were prepared using the solvents, and each experiment was repeated twice.

8. Statistical analysis

The data were shown as mean ± SD. The ED50 value which means the 50% inhibition of cell growth was calculated by using the R^2 equation. A P value < 0.05 was considered significant.

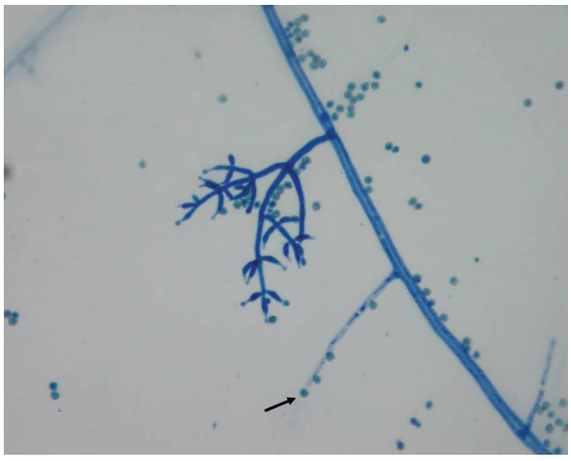


Fig 2. *Trichoderma* sp. grown on PDA for one week at room temperature: Angle of secondary branches attaching to the primary branches and phialides arise from conidiophores near 90°. Conidia are subglobose shape (arrow). Observed under light microscope at 400x magnification.

RESULTS

1.1. Endophytic fungus isolate

The colony of the isolate grew rapidly in 5 days on PDA at room temperature. By using macroscopic and microscopic morphology as shown in Fig 2, only one type of fungus was isolated. Young conidia with yellow pigmentation were first seen on media after 48 h. They form abundantly around the margin of the colony in cottony. The mycelium nearly filled the Petri dish with dense, confluent blue-green pustules of mature conidia forming in the center of the colony and subsequent conidia developing toward the margin; no diffusing pigment or distinctive odor noted. It was septate hyaline hyphae. Conidiophores were hyaline and lateral side branched pyramidal arrangement. Phialides were hyaline, flask-shaped, and arranged in clusters. They were attached to the conidiophores at right angles. Conidia were green ellipsoid at the tips of the phialides. It fits the description of *Trichoderma* species, described by Persoon ex Gray (1801).[11,12,13]

1.2. Effects of crude broth extract on the growth of cancer cells

The antiproliferative activities of crude broth extract were determined and found that no ED50 value was obtained. Therefore, the crude broth extract exhibited no antiproliferative effect on any cancer cell line in this experiment.

1.3. Effect of the extract on bacteria and *Candida*

The endophytes extracted in this study did not have antibacterial and anticandidal activities. All yeasts grew well with the extract after incubation. The results of the disc diffusion test show absence of inhibition zones for both *E.coli* and *S.aureus* experiments.

DISCUSSION

The purpose of this study was to investigate the antiproliferative and antimicrobial activities of endophytic fungus isolated from the stems of *Erycibe ellipticlimba* Merr.&Chun. From macroscopic and microscopic morphology, the only type of fungus obtained from the isolation was *Trichoderma* sp.

Trichoderma sp. is an endophytic fungus that is widely distributed in the soil, plant material, decaying vegetation, and wood. Many endophytic fungi isolated from the inner bark segments of many cultured medicinal plants were host and tissue specific and some *Trichoderma* spp. such as *Trichoderma reesei* has been reported to be able to produce some immunoreactive proteins.[14,15,16] Usually, the secondary metabolites of the fungus is secreted into the culture medium, therefore, the crude broth extract was used in this study to assess the antiproliferative and antimicrobial activities. However, the results revealed that the secreted metabolites of this fungus did not show any growth inhibitory effect on human cancer cells and microorganisms. Whereas some of the endophytic fungi such as *Paecilomyces* sp. had antitumor and antifungal activities, the presence of these activities of *Trichoderma* sp. had never been reported the same as in this study.[17] More importantly, *in vitro* culture of the fungus might possibly not synthesize an active compound and extraction processes might reduce the active and specific compounds which occurred in the endophytic fungus.

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CONCLUSION

Based on this study, the antiproliferative activities against several human cancer cell lines of *Erycibe elliptilimba*, Merr.&Chun. extract in recent reports may depend upon the active compounds in the plant itself.[3,4]

ACKNOWLEDGMENTS

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