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RESEARCH ARTICLE

Bioactivities of A Major Compound from *Arthrinium rasikravindrae* An Endophytic Fungus of *Coleus amboinicus* Lour.

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Bioactivities of A Major Compound from Arthrinium rasikravindrae An Endophytic Fungus of Coleus amboinicus Lour.

SUMMARY

Many studies reported the ability of endophytic fungi to produce various bioactive compounds having therapeutic values. An endophytic fungus identified as Arthrinium rasikravindrae was isolated from the stem of Coleus amboinicus Lour. This study examined cytotoxic and antimicrobial activities of a major compound isolated from ethyl acetate extract of the fungus fermentation broth. Cytotoxic activities were conducted using MTT assay against T47D, MCF-7, WiDr, 3T3, and Vero cells. IC50 values against Staphylococcus aureus and Escherichia coli were used as the parameters for determining antimicrobial activities. The isolated compound appeared as a single peak in HPLC chromatogram (98.55 %), displayed the highest cytotoxic activity on WiDr cells (IC50 35.03 ± 2.08 µg/mL) and antimicrobial activities against S. aureus (IC50 232.10 ± 1.20 µg/mL) and E. coli (243.59 ± 1.32 µg/mL). Analysis of the UV spectrum and TLC data generated by various detection reagents revealed that the compound was predicted as an N-containing substance having conjugated double bonds.

Key Words: *Coleus amboinicus Lour., cytotoxicity, antimicrobial, Arthrinium rasikravindrae, endophyte, fungus.*

Coleus amboinicus Lour'in Endofitik Mantarı Arthrinium rasikravindrae'den Elde Edilen Majör Bileşiğin Biyoaktiviteleri

ÖZ

Birçok çalışma, endofitik mantarların terapötik değerlere sahip çeşitli biyoaktif bileşikler üretme kabiliyetini bildirmiştir. Arthrinium rasikravindrae olarak tanımlanan bir endofitik mantar Coleus amboinicus Lour'in gövdesinden izole edilmiştir. Bu çalışmada, mantar fermantasyon suyunun etil asetat ekstresinden izole edilen ana bileşiğin sitotoksik ve antimikrobiyal aktiviteleri incelenmiştir. Sitotoksik aktivite testleri, T47D, MCF-7, WiDr, 3T3 ve Vero hücre hatlarına karşı MTT deneyi kullanılarak gerçekleştirilmiştir. Antimikrobiyal aktivitelerin belirlenmesinde Staphylococcus aureus ve Escherichia coli'ye karşı IC50 değerleri hesaplanmıştır. İzole edilen bileşik, HPLC kromatogramında (%98,55) tek bir pik vermiştir, WiDr hücreleri üzerinde en yüksek sitotoksik aktiviteye (IC50 35,03 ± 2,08 µg/mL) sahip olduğu belirlenmiş, S. aureus (IC50 232,10 ± 1,20 µg/mL) ve E. coli'ye (243,59 ± 1,32 µg/mL) karşı antimikrobiyal aktivite göstermiştir. Farklı reaktifler ile oluşturulan UV spektrumları ve İTK analizleri yorumlandığında bileşiğin, konjuge çift bağlara sahip N-içeren bir madde olduğu tahmin edilmektedir.

Anahtar Kelimeler: *Coleus amboinicus Lour., sitotoksisite, antimikrobiyal, Arthrinium rasikravindrae, endofit, mantar.*

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INTRODUCTION

Medicinal plants had been explored for many years for their potential sources of bioactive substances such as anti-microbial and anti-cancer agents (Khameneh et al., 2019; Swanepoel et al., 2019). Recently, many explored the potential of endophytic fungi inhabiting living plant tissue, including medicinal plants' tissues, as a source of bioactive compounds (Hazalin et al., 2009; Ran et al., 2017; Kumari et al., 2018; Leylaie and Zafari, 2018; Senthil Kumar et al., 2019). Coleus amboinicus Lour is a medicinal herb traditionally used as lalapogoe among Batakneese in Indonesia (Damanik et al., 2006). The leaves' aqueous extract of this plant had been shown to have anti-malarial activities in mice (Periyanayagam et al., 2008), while the essential oils were reported to have antimicrobial activities (Alankararao et al., 1991). The leaves also contained a high concentration of phenolic acids, rosmarinic acid, flavonoids, diterpenes, and linolenic acid (Yanza et al., 2018). Plant-derived phenolic compounds were reported to retard the growth of cancer in vitro, preclinical, and epidemiological studies (Anantharaju et al., 2016; Han et al., 2019). The ability of the fractions of leaves' ethyl acetate extract of *C. amboinicus* containing flavonoid in inhibiting the growth of several cancer cell lines was reported (Hasibuan et al., 2019). The plant stem methanolic extract was testified to have antioxidant and antibacterial activities. It also showed antiplatelet aggregation and antiproliferative activities against several cancer cell lines (Bhatt et al., 2013). This study was aimed to explore the potential of an endophytic fungus residing in the stem of *C. amboinicus* in producing bioactive molecules as anti-microbial and cytotoxic agents against cancer.

MATERIALS AND METHODS

Materials

The pure culture of *Arthrinium rasikravindrae* was isolated from the stems of *C. amboinicus* (family Lamiaceae). The plant was collected from Medicinal Plant Garden, Faculty of Pharmacy, Universitas Gadjah Mada. The plant species identification was conducted (by Abdul Razaq Chasani, Ph.D.). A plant herbarium was deposited for future reference in the Pharmaceutical Biology Department, Faculty of Pharmacy, Universitas Gadjah Mada; reference number 014745. The culture was grown on Potato Dextrose Agar (PDA) plates without antibiotics, maintained for routine culture, and stored in glycerol stock for culture collection at the Department of Pharmaceutical Biology, Faculty of Pharmacy Universitas Gadjah Mada. Potato Dextrose Agar (PDA), Dextrose, Nutrient Agar (NA), Mueller Hinton were purchased from Oxoid. Silica gel F254, Silica gel 60 PF254 containing gypsum, n-hexane, chloroform, ethyl acetate, methanol, acetic acid glacial, DMSO (dimethyl sulfoxide) (Merck). RPMI 1640, Fetal Bovine Serum, Sodium bicarbonate, L-glutamine, Penicillin-Streptomycin, Fungizone(Gibco). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Invitrogen). Equipment: HPLC (Waters 2998), UV-Vis spectrophotometer (Shimadzu).

Isolation and Identification of Endophytic Fungus

Isolation of the endophytic fungus was conducted using the protocol used by Ding et al. (2010) with some modification (Ding et al., 2010). In brief, the stems were washed with running tap water and surface-sterilized using 70% ethanol for 1 minute. After immersion on 5% sodium hypochlorite for 3 minutes, the stems were drained and re-soaked in 70% ethanol for 30 seconds. Following three times washing with sterile distilled water, the stems were surface-dried onto sterile filter paper and cut 1 cm long aseptically. The surface-sterilized stems were placed onto PDA plates containing 30 µg/mL streptomycin. The plates were incubated at 25°C for 2 to 3 days. The hyphal tip of the endophytic fungus that comes out of the stem was moved into new PDA plates containing 30 µg/mL streptomycin and further incubated for 10 – 14 days. The pure culture was obtained by subculturing endophytic fungus on PDA plates without antibiotics. This culture was maintained for the Pharmaceutical Biology Department, Faculty of Pharmacy Universitas Gadjah Mada collection. Identification of endophytic fungus was conducted by PCR amplification using ITS Primer 4: 5`-- TCC TCC GCT TAT TGA TAT GC – 3`, and ITS Primer 5: 5`--GGA AGT AAA AGT CGT AAC AAG G –3`. PCR product was purified and precipitated using Polyethylene Glycol precipitation technique (Hiraishi et al., 1995), continued by cycle sequencing. The result was re-purified by the ethanol purification method. Sequencing was conducted using

an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer-Applied Biosystems). Trimming and assembling the sequenced data was performed using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.htmL) followed by BLAST alignment using genomic data inDDBJ/ DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/)or NCBI/ National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) to analyze homology/ similarity.

Fermentation and Culture Medium Extraction

The endophytic fungus was inoculated on 500 mL culture vessels containing potato dextrose broth medium. The culture was incubated on a shaker incubator at 25°C for 14 days at 160 rpm. Mycelium was separated from the supernatant using What¬man filter paper, followed by centrifugation at 4000 rpm for 5 min to obtain mycelium free supernatant. The supernatant was extracted using ethyl acetate (ratio 1:1 v/v) three times to obtain ethyl acetate fractions. The solvent was evaporated, resulted in ethyl acetate extract. The components of the extract were compared with the ones of *C. amboinicus* stem ethyl acetate extract. The mycelia were dried and copped, followed by 24 h maceration using ethyl acetate. The solvent was evaporated to obtain mycelia extract.

Isolation of Bioactive Compound

Ethyl acetate extract was separated using column chromatography [stationary phase = silica gel 60 F254; mobile phase = gradient concentration of chloroform: ethyl acetate]. Fractions having similar TLC profiles were combined and tested for cytotoxic activities. Active fractions were further separated with preparative thin-layer chromatography [stationary phase = silica gel 60 PF254; mobile phase = n-hexane: chloroform: ethyl acetate (1:5:1 v/v). A major positive UV254 compound within the fraction was isolated and tested for its purity using TLC. A single peak on the HPLC profile indicated a pure compound. The predicted chemical group of the compound was established by analyzing TLC profiles and UV data.

Cytotoxic Activity

T47D (human breast cancer cells), MCF-7 (human breast cancer cells), WiDr (human colon cancer cells), 3T3 (mouse embryonic fibroblast cells), and Vero (African green monkey kidney epithelial) cells were cultured in RPMI 1640 containing 10% FBS, 1% penicillin/streptomycin and 1% fungizone. Cultures were cultivated in a CO2 incubator at a temperature of 37°C, 95% relative humidity, and 5% CO2. 5x103 cells in 100 µL media were inoculated into 96 well plates and incubated for 48 hours to reach 70% – 80% confluent, followed by adding 100 µL of a series concentration of the isolated compound (final concentration of 6.25 – 200 µg/mL for testing on T47D, MCF7, and WiDR cells; 9.37 – 600 µg/mL for testing on 3T3, and Vero cells). Doxorubicin, a conventional chemotherapeutic agent, was used as the positive control, and DMSO was used as solvent control. Doxorubicin at the concentration ranges of 0.31 – 20.0 µg/mL were used for testing on T47D, MCF7, and WiDR cells, while the concentration ranges of 62.50 – 2000 µg/mL were used for testing on 3T3 and Vero cells. The plates were further incubated for 24 hours, followed by gentle washing with 1X PBS. After incubation with 100 µL of 0.5 mg/mL MTT, the plates were incubated for 4 hours at 37oC, and 100 µL of SDS 10% was added to end the reaction. Following overnight incubation, the absorbances were determined using a microplate reader (Biorad) at 595 nm. IC50 values were calculated based on the dose-response curve of cells treated with various concentrations of the isolated compounds.

Antimicrobial Testing

Testing for anti-microbial activity was conducted using a modified microdilution method (Muhammad et al., 2003; da Silva Filho et al., 2008) against Escherichia coli (ATCC 11229) and Staphylococcus aureus (ATCC 25923). Testing microorganisms with densities equal to 0.5 McFarland standard were diluted (1:10) with Mueller Hinton and transferred triplicate into a 96-well plate. Serially diluted samples were transferred into 96 well plates containing testing microorganisms to a final concentration of 2000 to 15.6 µg/mL. Controls of microbial growth, solvent, and media were included in the testing plate. Streptomycin was used as a positive control. The plates were incubated at 37o C overnight and read at 595 nm using a microplate reader (Biorad). IC50, the concentration that inhibited 50% of microbial

growth, were determined from plotting percent growth and concentration of sample tested.

RESULTS AND DISCUSSION

The stem of Coleus amboinicus Lour was reported to contain antioxidant compounds. Its methanolic extract exhibited an antiproliferative effect against several cancer cell lines (Bhatt et al., 2013). The endophytic fungi isolated from *C. amboinicus* stem resulted in the finding of Arthrinium rasikravindrae, phylum Ascomycota. The same species were found in Brassica capestris as well as bamboo leaves. The same genus was found as phylloplane fungus in chili plants (Rana et al., 2017; Wang et al., 2018).

It has been observed that some endophytes produced bioactive metabolites that may be the same as those of the host plants (Strobel et al., 1996; Pu et al., 2013; Ran et al., 2017; Tan et al., 2018) or different (Gangadevi and Muthumary, 2008; Rajendran et al., 2013; Gill and Vasundhara, 2019). Based on preliminary detection using TLC and UV light, this study found that metabolites within ethyl acetate extract of *A. rasikravindrae* fermentation broth were different from those in ethyl acetate extract of the stem of *C. amboinicus*. Various UV254 positive compounds were observed in broth extracts that were absent in the stem extracts (Figure 1). The major metabolites within broth extract, however, were similar to those within mycelia. The data indicated that the metabolites might be produced in response to their environment as well as their function as fungal protection (Tripathi and Joshi, 2019).

Bioassay-guided isolation of the bioactive compound within the broth ethyl acetate extract resulted in the finding of a compound which exhibited cytotoxic activity against T47D cells. Liquid chromatography analysis of this compound demonstrated a 98.55 % level of purity with a retention time of 3.739 (Figure 2). Further bioactivity screening against several other cancer cell lines showed cytotoxic activity with the lowest IC50 value of 35.03 ± 2.08 µg/mL against WiDR (Table 1). Antibacterial testing of the compound showed an IC50 value of 232.10 ± 1.20 against *S. aureus* and 243.59 ± 1.32 µg/mL against *E. coli* as compared to control streptomycin, which exhibited an IC50 value of 0.81 ± 0.02 µg/mL.

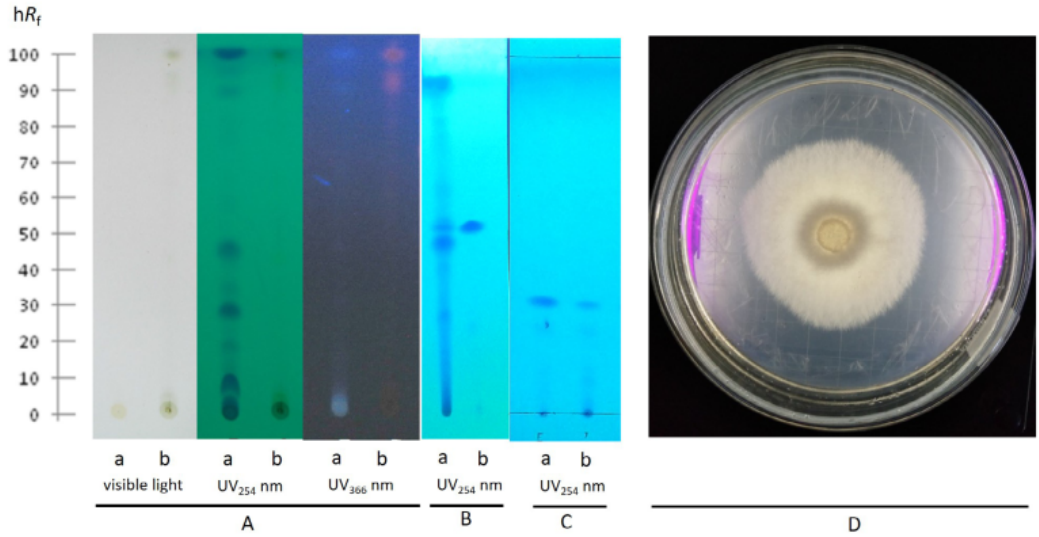

Figure 1. TLC profiles of ethyl acetate extract of fermentation broth of *A. rasikravindrae* endophytic fungus (a) and stem ethyl acetate extract (b) detected with visible light, UV_{254} nm and UV_{366} nm light (from left to the right). [stationary phase = silica gel F_{254}; mobile phase = chloroform : ethyl acetate (7:1 v/v)] (A). TLC profiles of fermentation broth ethyl acetate extract (a) and isolated compound (b) detected with UV_{254} nm [stationary phase = silica gel F_{254}; mobile phase = chloroform : ethyl acetate : acetic glacial acid (15 :5:1 v/v) (B). TLC profiles of ethyl acetate extract from fermentation broth (a) and mycelia (b) detected with UV_{254} nm [stationary phase = silica gel F_{254}; mobile phase = chloroform : ethyl acetate (7:1 v/v) (C). *A. rasikravindrae* grown on a PDA plate (D).

Table 1. IC_{50} values of the compound against several cell lines. Data represent the mean of IC_{50} ± SD (n = 3).

Cell lines	Compound	Doxorubicin
	IC_{50} ± SD (µg/mL)	IC_{50} ± SD (µg/mL)
T47D	45.87 ± 1.42	4.86 ± 0.21
MCF7	117.93 ± 0.87	0.95 ± 0.07
WiDR	35.03 ± 2.08	3.19 ± 0.27
3T3	411.97 ± 17.43	192.93 ± 4.98
Vero	141.67 ± 8.9	297.59 ± 10.1

Bio-screening activity indicated that this compound exhibited selective cytotoxicity with the lowest IC50 observed against colon cancer cell line WiDr, followed by breast cancer cell line T47D. Testing against another type of breast cancer cell line MCF7 showed less potency with IC50 above 100 µg/mL. T47D and MCF7 cell lines are both breast cancer cell lines in which they are different in p53 characteristic; T47D is a p53 missense mutant cell line, while MCF7 is a cell line that expresses p53 wild type (Lim et al., 2009). The IC50 value difference between the T47D cell line and MCF7 cell line showed the different mechanisms to induce cell death, which may be p53 independent. Besides, higher IC50 values were observed when the compound was tested on normal cells (3T3 and Vero cell lines), indicating the selectivity of this compound towards cancer cell lines. Antibacterial testing of the compound against *S.aureus* and *E.coli* exhibited IC50 values of almost 300 times higher than streptomycin control showing less sensitivity of these microorganisms towards tested compounds (Cos et al., 2006).

Preliminary characterization based on TLC profiles detected using spray reagents showed that the isolated compound positively reacted with all detection reagents except FeCl3 (Figure 2). The absence of colored spots upon FeCl3 detection showed that the compound did not have a phenol group. The positive reaction with 2,4-DNPH, indicating the presence of a carbonyl functional group of aldehydes or ketones (Krupadanam et al., 2001). While cerium (IV) sulfate functioned as a universal reagent for detecting organic compounds, orange-brown positive reaction with Dragendorff suggested that the compound may be a class of alkaloid organic chemical, heterocyclic nitrogen compound or that having quaternary amine. A violet-blue spot upon spraying with vanillin sulphuric acid suggested the presence of terpenoid. (Wagner and Bladt, 1996; Aniszewski, 2015). Ultraviolet spectrum (MeOH) showed λ maximum absorption at 222.9 nm (Figure 2) suggested that the compound may have a conjugated double bond system (Singh and Singh, 2018). A further experiment is needed to characterize the chemical structure of the bioactive compound completely.

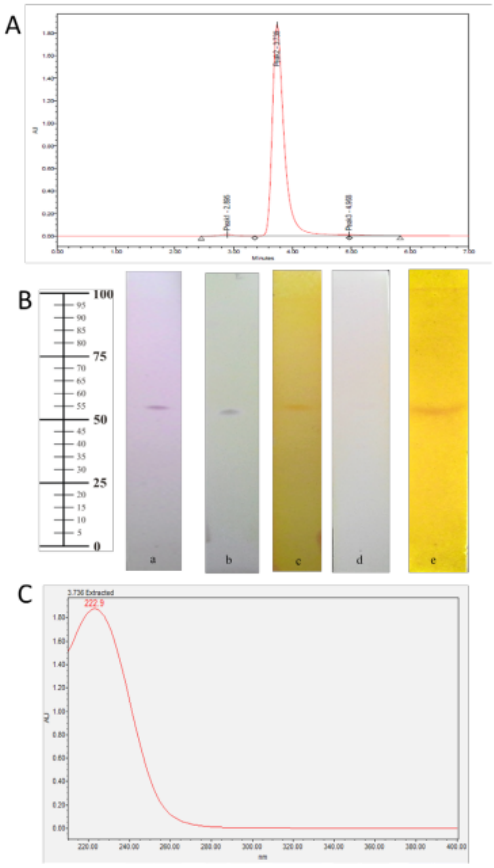

Figure 2. Liquid Chromatography profile of isolated compound. System: reverse phase chromatography. Mobile phase: methanol 100%. Stationary phase: c18. Flow rate: 0.5 ml/minute. λ = 222.9 nm (A). TLC profiles of isolated compound detected using various spray reagents vanillin-sulphuric acid (a), Cerium (IV) sulphate (b), 2,4-dinitrofenilhidrazin (2,4-Dinitrophenylhydrazine) (c), $FeCl_3$ (d), Dragendorff (e) (B). [stationary phase = silica gel F_{254}; mobile phase = chloroform : ethyl acetate : acetic glacial acid (3 :1:0.15 v/v). UV (MeOH) spectrum of isolated compound (C).

CONCLUSION

The present study reported the isolation and characterization of a major compound from an endophytic fungus *A. rasikravindrae* isolated from the stem of *C. amboinicus*. The compound exhibited moderate cytotoxicity to T47D and WiDr cancer cell lines but less active towards microorganisms tested.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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AUTHOR CONTRIBUTION STATEMENT

Concept and experimental design, data acquisition, cytotoxicity study, writing and final approval of manuscript (Astuti P.), isolation of bioactive compound, cytotoxicity experiment, chromatography analysis, writing and final approval of manuscript (Pratoko D.K.), antimicrobial assay study, writing and final approval of manuscript (Rollando R.), TLC analysis, writing and final approval of manuscript (Nugroho G.W.), in isolation of bioactive compound, spectra analysis, writing and final approval of manuscript (Wahyuono S.), bioactivity and spectra analysis, writing and final approval of manuscript (Hertiani T., Nurrocmad A.).

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