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Research Article

Evaluation of Antibacterial and Antioxidant Activity of Endophytic Fungi Isolated from *CAPSICUM ANNUUM* L. and *ALLIUM CEPA* L.

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ABSTRACT

Objective: The aims of this study were to identify the endophytic fungi from *Capsicum annuum* L. and *Allium cepa* L., to determine antioxidant and antimicrobial activity of ethyl acetate extract of endophytic fungi isolated from *C. annuum* and *A. cepa*. **Methods:** Endophytic fungi was isolated with potato dextrose agar (PDA) from fruits of *C. annuum*and bulbs of *A. cepa*. Isolate of endophytic fungi was molecular identified to know the species or genus. Cultivation was carried out on rice media, 4 weeks on room temperature and the extraction by maceration using ethyl acetate. Antioxidant activity were tested by DPPH method. While antibacterial activity was tested by disk diffusion methods and microdilution methods. **Results:** Five isolates of endophytic fungi from red and green fruits of *C. annuum* and bulb of *A. cepa* have been isolated and the species or the genus have been confirmed. KCM 1 and KCM 2 isolates endophytic fungi from the red fruits of *C. annuum* were confirmed as *Diaporthe sp* and *Chaetomium globosum*. The KCH 1 isolate from green fruits of the *C. annuum* was confirmed as *Trametes hirsuta*. The KBM 1 and KBM 2 isolates from *A. cepa* were confirmed as *Schizophyllum commune* and *Phlebia sp*. The highest antioxidant and antibacterial activity was exposed by ethyl acetate extract of *S. commune*. **Conclusion:** Five isolates endophytic fungi from *C. annuum* and *A. cepa* were *Diaporthe sp, C. globosum, T. hirsuta, S. commune* and *Phlebia sp.* Ethyl acetate extract of *S.commune* gave highest antioxidant and antibacterial activity.

Key words: Antimicrobial, *Chaetomium globosum,* Endophytic fungus, Onion, Red chili, *Schizophyllum commune.*

INTRODUCTION

Endophytic fungi are microorganism that live in internal plant tissue. This fungus doesn't cause negative effect on the host plant. Almost all of plants have endophytic fungi. The plant can have one or more species of endophytic fungi.[1]

In present, so many researchers interest with endophytic fungi because endophytic fungi can produce secondary metabolites likes its host plant.[2] Endophytic fungi is another way to search secondary metabolites from medicinal plant, that have activities like anti-inflammatory, antimicrobial, and anticancer properties.[3,4] This topic can be solution for a crop that is barely being found anymore and will save costs.

In Indonesia, *Capsicum annuum* L. that namely as "cabe keriting". These plant is used as kitchen spices, that have a spicy flavour. The other plant is *Allium cepa* L. or "bawang merah" which used as a flavoring. Both of this plants, not only as a spices, but are used as traditional medicine. In a traditional medicines, fruits of *C.annuum* was used as a therapy for rhematism, arthritis, abdominal discomfort ability and irritation.[5,6] While *A.cepa* was used as a therapy for diarrhea diseases by the china's people.[7] Capsaicin is one of the compounds in *C.annuum,* which had potential anti-inflammatory activity and quite expensive in the market. Related research regarding secondary metabolites of endophytic bacteria, reported that *Acinetobacter baumannii* was successfully isolated from *C. annuum* and it had antioxidant activity.[8]

MATERIALS AND METHODS

Materials collection

C. annuum and *A. cepa* were collected from Lembang, Bandung, West Java, Indonesia, and identified by Herbarium Bandungense, School of Life and Science Technology, Bandung Institute of Technology, potato dextrose agar (Himedia), potato dextrose broth (Himedia), Mueller Hinton agar (Himedia), Mueller Hinton broth (Himedia), blank disc, chloramphenicol disc, *Stphylococcus aureus*, *Escheria coli*, *Basillus subtilis*, *Pseudomonas aeuruginosa*, DPPH, methanol (Merck), DMSO and ethyl acetate.

Isolation of endophytic fungi

The isolation method of endophytic fungi was carried out from previous research.[9] The samples washed by running water and cut into 0.5 cm sections. For surface sterilization, the bulbs of *A. cepa* and fruits of *C. annuum* submerged in ethanol 70% v/v for 2 min, and submerged in 1% natrium hypochlorite solution for 3 min and the last submerged in ethanol 70% v/v for 30s. After surface sterilization, the sample was grown on PDA and incubated at 25°C for 2 weeks until the endophytic fungi isolated. Chloramphenicol is added to PDA media to reduce or inhibit growth of bacteria. Bulbs of *A. cepa* and fruits of *C. annuum* that were not surface sterilized used as a negative control. Producing pure isolates endophytic fungi were conducted by subculture in a same media and condition. Then pure isolates endophytic fungi from

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bulbs of *A. cepa* and fruits of *C. annuum* were molecular identified to obtain the most suitable species relatedness.

Macroscopic test

Macroscopic test was carried out to see the morphological characteristic of endophytic fungi that was isolated from bulbs of *A. cepa* and fruits *C. annuum.* Morphological characteristic will show the color, shape, growth rate and surface texture of colony.

Molecular identification

Genomic DNA extraction with Quick –DNA Fungal Miniprep Kit. The pure endophytic fungi were molecular identified using the internal transcribed spacer (ITS) region. Polimerase chain reaction (PCR) amplification contained MyTaq HS Red Mix with ITS 1 and ITS 4.[10] Then the product PCR was sequensed on 1st BASE, Malaysia. The sequence was subjected to aligment using the Basic Local Aligment Search Tool (BLAST) program on National Center for Biotechnology Information (NCBI).

Cultivation and extraction of endophytic fungi

Endophytic fungi cultivation was conducted using rice media for ±4 weeks with dark condition and room temparature (25 °C).[9] Observation was done every day to prevent contamination from other microbes.

Then the cultivation results were extracted by maceration using ethyl acetate. Maceration was performed 1x24 hrs, in three replication. The ethyl acetate extracts were evaporate using rotary evaporator to obtain thick extracts. Ethyl acetate extracts of endophytic fungi from bulbs of *A. cepa* and fruits of *C. annuum* were carried out for bioactivity test.

Antibacterial activity test[11]

Before being used, bacterium was grown separately in Meuller Hinton Agar (MHA) and incubated at 37°C for 24 h. These cultures were used for antimicrobial assay by modified agar disc diffusion method of Kirby and Bauer. Single colony of the respective testing bacterium was transferred into MHB medium and incubated for 24 h. 100 µl culture suspension of testing bacterium with 25% transmittans was pipette onto MHA medium. Each ethyl acetate extract was prepared to concentration of 200, 100, 50 mg/ml in DMSO. Each 10 µl of extract above was dropped onto blank disc (6 mm diameter) and carefully placed on the culture suspensions for cultivation plate. Positive control disc contained chloramphenicol 30 µg/disc and DMSO was used as a negative control. Each plate was incubated at 37°C for 18 - 24 h. Inhibition zones (including the diameter of disc) were measured and recorded.

Microdilution test was conducted to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) using Clinical and Laboratory Standards Institute(CLSI).[12] This method was performed using the a 96-well microplate. Two wells were filled as a control, 100 µl MHB and 100 l bacteria testing with 100 µl MHB. Ten wells were filled by a sample with a series concentrations 100 µl/well with 100 µl MHB. In this study, 100 mg/ml was applied as highest concentration. The a 96-well microplate which have been filled was incubated at 37°C for 18 -24 h. In MBC determination, MHA was used as a media and concentration of sample using MIC result. The concentration that exhibited no apparent bacterial growth, was used as sample. It were streaked on the surface of the agar with an sterile needle. After that, the petri dish was incubated at 37°C for 18 -24 h. The lowest concentration, which did not grow on the subculture, was recorded as the MBC.[13]

Antioxidant activity test[14]

30 mg DPPH free radical was dissolved in 1000 ml methanol then scanned between 400-800 nm to get maximum absorption wavelength, then the maximum wavelength was used to investigate the absorbance of sample after being mixed with DPPH solution. Each sample diluted with methanol to make the concentration of ethyl acetate extract 150, 100, 50, 25, 12,5, 6,25, 3, 1,5 µg/ml, as well as control ascorbic acid were prepared at the concentration of 6, 5, 4, 3, 2 and 1 µg/ml. Methanol was used as a blank and DPPH solution as a control.[15] Each extract as well their dilution was pipetted 2 ml and mixed with 2 ml DPPH solution. The absorbance of mixing solutions was measured 30 min after incubation. The data obtained were processed for obtaining regression equation to evaluate IC_{50}. Meanwhile Antioxidant Activity Index (AAI) was calculated by final DPPH concentration divide by $IC_{50.}$[16]

RESULTS AND DISCUSSION

Macroscopic test

In this study, two endophytic fungi have been isolated from bulbs of *A. cepa* (KBM 1 and KBM 2), two endophytic fungi have been isolated from red fruits of *C. annuum* (KCM1 and KCM 2) and one endophytic fungi had been isolated from green fruits *C. Annuum* (KCH 1). The macroscopic test was presented in the following data (Figure 1 and Table 1).

Moleculer identification

The result of molecular identification showed that KCM 1 had > 99% similarity with *Diaporthe sp*., KCM 2 had > 99% similarity with *Chetomium globosum*, KCH 1 had > 99% similarity with *Trametes hirsuta*, KBM 1 had > 99% similarity with *Schizophyllum commune* and KBM 2 had > 99% similarity with *Phlebia sp*.

The endophytic fungi which have been isolated were not new species, such as *T. hirsuta* had been isolated from *Podophyllumhexandrum,*[17] *S. commune* from *Cannabissativa* and *C. globosum* from *Picrorhiza kurroa.*[17,18] But in both plants (*A. cepa* and *C. annuum*) the types of endophytic fungi were new types.

The endophytic fungi isolated from *C. annuum* was *Alternaria alternata* and produced capsaisin.[19] In addition, from the *C. annnum*, endophytic bacteria had also been isolated, *Acinobacter baumannii*.[8]

Antibacterial activity

Antibacterial activity was tested by Kirby-Breur method to get the diameter inhibition zone. Agar disk diffusion was used because offer many advantages over other methods: simplicity, low cost and the ability to test enormous numbers of microorganisms and antimicrobial agents and the ease to interperet results provided. However, this method is not appropriate to determine the MIC.[20] In this study, to know the MIC and MBC were used microdilution methods. The results were exposed in table 3.

Based on the results expressed that isolate of endophytic fungi from bulbs of *A.cepa* and fruits of *C. annuum* can inhibit the growth of *S. aureus, B. subtilis*, and *P. aureuginosa* bacteria. The classification of strength antibacterial activity is classified as follows: very strong (>20 mm), strong (10-20 mm), medium (5-10 mm) and weak (<5 mm).[21] Ethyl acetate extract of *S. commune, Phlebia sp, T. hirsuta and Diaporthe sp* at the 5% concentration showed strong antibacterial activity against Gram positive bacteria *S. aureus* and *B. subtilus*. The previous research stated that *S. commune* from *Veronica anthelmitica* had antibacterial and antifungal activity against *E.coli*, and *C. albicans.*[22]

Classification of antibacterial activity with the following ranges: MIC values are <100 µg/ml is a high antibacterial activity, 100 – 500 µg/ml a moderate antibacterial activity, 500 – 1000 µg/ml a weak antibacterial activity, and > 1000 µg/ml no antibacterial effect.[23,24] Based on MIC results and classification of antibacterial activity, were known that *T. hirsuta* and *C. globosum* had the highest MIC value (0.39 mg/ml) with

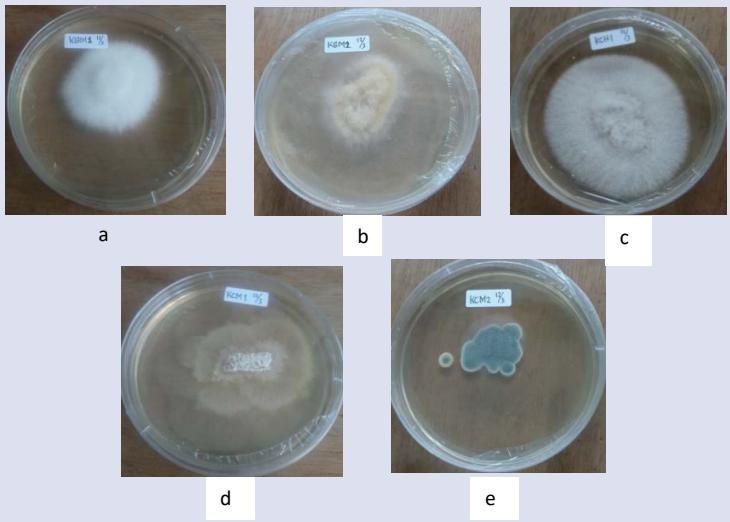

Figure 1: Appearance of endophytic fungi colony isolated from *C. annuum* and *A. cepa* on PDA medium at 25°C. a) KBM 1 b) KBM 2 c) KCH 1 d) KCM 1 and e) KCM 2.

Table 1: Macroscopic data of endophytic fungi from C. *annuum* and A. *cepa* on PDA medium at 25 °C after 6 days.

Macroscopic characteristic	Morphotype				
	KCM 1	KCM2	KBM 1	KBM 2	KCH 1
Color	White	Green	White	Orange	White
Shape	Circular	Irregular	Circular	Circular	Circular
Growth (cm)	6.0 ± 0.1	5.0 ± 0.3	7.0 ± 0.1	9.0 ± 0.1	8.5 ± 0.6
Surface texture	Cottony	Sporaes	Cottony	Cottony	Cottony

Table 2: Diameter inhibition zone of ethyl acetate extract of endophytic fungi from A. *cepa* and C. *annuum*.

Ethyl acetate extract	Diameter inhibition zone (mm) ± standrad deviation (SD)											
	S. aureus			B. subtilis			E.coli			P.auroginosa		
	20%	10%	5%	20%	10%	5%	20%	10%	5%	20%	10%	5%
S. commune	14.50 ± 1.25	12.52 ± 0.70	11.78 ± 0.91	18.80 ± 1.87	18.73 ± 2.2	17.28 ± 6.6	-	-	-	8.43 ± 1.83	7.3 ± 0.69	7.3 ± 1.61
Phlebia sp	-	-	-	12.07 ± 2.95	12.2 ± 2.43	10.08 ± 0.73	-	-	-	7.63 ± 2.17	-	-
T. hirsuta	-	-	-	13.6 ± 3.87	13.77 ± 1.19	10.43 ± 0.94	-	-	-	-	-	-
Diaporthe sp	8.5 ± 1.91	6.6 ± 1.13	6.75 ± 0.41	16.52 ± 2.37	13.25 ± 2.95	13.25 ± 2.95	-	-	-	7.6 ± 1.01	7.1 ± 0.95	6.57 ± 0.55
C. globosum	10.08 ± 0.35	10.08 ± 0.35	7.65 ± 0.9	10.77 ± 0.74	10.33 ± 0.58	10.67 ± 1.15	-	-	-	7.23 ± 0.32	-	-
Chloramphenicol	24.20			32.53			23.9			16.50		
DSMO	-			-			-			-		

Table 3: MIC and MBC of ethyl acetate extract of endophytic fungi from A. *cepa* and C.*annuum*.

Ethyl acetate extract	MIC (mg/ml)				MBC (mg/ml)			
	S. aureus	B. subtilis	E. coli	P. aeuroginosa	S. aureus	B. subtilis	E. coli	P. aeuroginosa
S. commune	6.25	0.78	12.5	12.5	25	0.78	12.5	12.5
Phlebia sp	3.12	0.39	25	100	50	0.39	50	50
T. Hirsuta	12.5	100	25	25	25	12.5	25	25
Diaporthe sp	25	12.50	50	6.25	25	6.25	25	6.25
C. globosum	25	0.39	50	3.12	25	0.78	12.5	6.25

Table 4: Antioxidant activity of ethyl acetate extract of endophytic fungi from A.*cepa* and C. *annuum*.

Ethyl acetate extract	IC_{50} (µg/ml)	AAI
S. commune	3.15 ± 0.88	5.00± 1.34
Phlebia sp	118.13 ± 15.36	0.13± 0.02
T. Hirsuta	142.25 ± 1.09	0.11± 0.00
Diaporthe sp	121.91 ± 10.32	0.12± 0.01
C. globosum	213.78 ± 11.67	0.07± 0.00
Ascorbic acid	1.36 ± 0.01	11.00± 0.04

moderate antibacterial activity against *B. subtilis*. While, *S. commune* had MIC value 0.78 mg/ml as weak antibacterial activity against *B. subtilis*.

Antioxidant activity

The antioxidant activities of the ethyl acetate extracts of endophytic fungi were evaluated by determining the IC_{50} values and Antioxidant Activity Index (AAI) of DPPH. The results of antioxidant activity were exposed in Table 4. Lower IC_{50} value indicated higher antioxidant activity. The lowest value of IC_{50} was found in ethyl acetate extract of *S. commune*, which had the highest antioxidant activity.

The ethyl acetate extract of *S. commune* exhibited IC_{50} and AAI value of 3.15 ± 0.88µg/ml and 5.00± 1.34, respectively. Based on literature, if IC_{50} value on DDPH test < 50 µg/ml was categorized as very strong antioxidant activity. It can be predicted that ethyl acetate extract of *S. commune* had very strong antioxidant activity. The same results if categorized by AAI value, the ethyl acetate of *S. commune* can be classified as very strong antioxidant. Based on the results of Rustamova's research (2020) which examined the antioxidant profile of *S. commune* from *Veronica anthelmintica*, expressed that it had antioxidant activity with IC_{50} value 55.21 ± 0.3µg/ml.[22] The host plant of the endophytic fungi *S. commune* in this present study was *A. cepa*, which had stronger antioxidant activity than the previous research. It can be suggested that *S. commune* form different host plant can be given different effect. Based on literature review, exposed that the *A. cepa* had very strong antioxidant activity. The other study stated that *A. cepa* contained phenolic compounds such as gallic acid, ferulic acid, kaempferol and quercetin. The compounds were known had strong antioxidant activity.[25]

CONCLUSION

Five of endophytic fungi have been isolated and molecular identified from *C. annuum* and *A. cepa*. Based on activity test (antioxidant and antibacterial activity), *S. commune* endophytic fungi which was isolated from *A. cepa* have the potency as antioxidant and antibacterial agent. In the future research will be continued in separation and purification to obtain one or more secondary metabolites which have antioxidant and or antibacterial activity.

ACKNOWLEDGEMENT

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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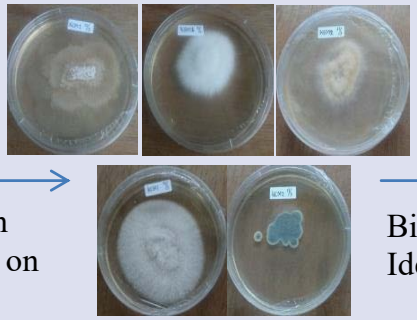
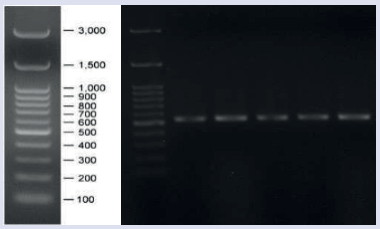
GRAPHICAL ABSTRACT

Isolation of fungi on PDA



Biomolecular Identification



Cultivation in Rice media 3 – 4 weeks

Extraction with ethyl acetate

Antioxidant activity test

Ethyl acetate extract	IC_{50} (µg/ml)	AAI
S. commune	3.15 ± 0.88	5.00 ± 1.34
Phlebia sp	118.13 ± 15.36	0.13 ± 0.02
T. Hirsuta	142.25 ± 1.09	0.11 ± 0.00
Diaporthe sp	121.91 ± 10.32	0.12 ± 0.01
C. globosum	213.78 ± 11.67	0.07 ± 0.00
Ascorbic acid	1.36 ± 0.01	11.00 ± 0.04

Antibacterial activity test

Ethyl acetate extract	MIC (mg/ml)				MBC (mg/ml)			
	S. aureus	*B. subtilis*	*E. coli*	*P. aeuroginosa*	*S. aureus*	*B. subtilis*	*E. coli*	*P. aeuroginosa*
S. commune	6.25	0.78	12.5	12.5	25	0.78	12.5	12.5
Phlebia sp	3.12	0.39	25	100	50	0.39	50	50
T. Hirsuta	12.5	100	25	25	25	12.5	25	25
Diaporthe sp	25	12.50	50	6.25	25	6.25	25	6.25
C. globosum	25	0.39	50	3.12	25	0.78	12.5	6.25

Ethyl acetate extract	Diameter inhibition zone (mm) ± standrad deviation (SD)											
	S. aureus			*B. subtilis*			*E.coli*			*P.auroginosa*		
	20%	10%	5%	20%	10%	5%	20%	10%	5%	20%	10%	5%
S. commune	14.50 ± 1.25	12.52 ± 0.70	11.78 ± 0.91	18.80 ± 1.87	18.73 ± 2.2	17.28 ± 6.6	-	-	-	8.43 ± 1.83	7.3 ± 0.69	7.3 ± 1.61
Phlebia sp	-	-	-	12.07 ± 2.95	12.2 ± 2.43	10.08 ± 0.73	-	-	-	7.63 ± 2.17	-	-
T. hirsuta	-	-	-	13.6 ± 3.87	13.77 ± 1.19	10.43 ± 0.94	-	-	-	-	-	-
Diaporthe sp	8.5 ± 1.91	6.6 ± 1.13	6.75 ± 0.41	16.52 ± 2.37	13.25 ± 2.95	13.25 ± 2.95	-	-	-	7.6 ± 1.01	7.1 ± 0.95	6.57 ± 0.55
C. globosum	10.08 ± 0.35	10.08 ± 0.35	7.65 ± 0.9	10.77 ± 0.74	10.33 ± 0.58	10.67 ± 1.15	-	-	-	7.23 ± 0.32	-	-
Chloramphenicol		24.20			32.53			23.9			16.50	
DSMO		-			-			-			-	

ABOUT AUTHORS

Sylvia Rizky Prima is Doctoral student in Institue Technology of Bandung and lecture in Faculty of Pharmacy, 17 August, 1945 Jakarta University. Develop work in phytochemical of natural material.

Elfahmi is Assoc. Professor in Departement of Pharmaceutical Biology, School of Pharmacy, Institue Technology of Bandung. Develop work in phytochemistry, pharmacognosy and biotechnology of medicinal plants.

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