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Research Paper

Potential of *Anabaena Azollae* Extract as Antimicrobial Agent For Paddy Crop Disease

Nuni Gofar[1]*, Andi Diana[2], Eka Setianingsih[2]

[1]*Department of Soil Science, Faculty of Agriculture, Sriwijaya University, Indonesia*
[2]*Department of Agroecotechnology, Faculty of Agriculture, Sriwijaya University, Indonesia*
***Corresponding author**: nigofar@unsri.ac.id*

Abstract
The research objective was to test antimicrobial activity from compound produced by A. azollae which symbiosis with Azolla sp. toward microbial test of *X. oryzae* and *Pyricularia oryzae* as cause of disease on paddy crop. Sampling of Azolla which symbiosis with *A. azollae* was done in Azolla cultivation pond at Faculty of Agriculture, Sriwijaya University. The available Azolla was consisted of two types, i.e. Azolla pinnata and *Azolla mycrophiylla*. The extraction of A. azollae from Azolla leaves was done by method of Ultrasound Assisted Extraction (UAE) using ethyl ester solvent and maseration method using methanol solvent. Test media for bacteria and fungus respectively are Mueller Hilton Agar (MHA) and Sabouraud Dextrose Agar (SDA). The diameter of produced clear zone is an indication of extract's inhibitory power toward bacteria or fungus. The different of inhibitory zone diameter is compared by using t-test. Analysis of active compounds on *Anabaena azollae* extract was done by using HPLC. Ethyl acetate or methanol extract of A. azollae which symbiosis with *A. pinnata* or *A. microphylla* was capable to inhibit the growth of X. oryzae bacterium and P. oryzae fungus. The dominant compounds containing within extract with probability more than 60% and area of more than 10% are consisted of phytol, hexadecanoate acid and 2-(tert-butyl)-4,6-dimethyl phenol.

Keywords
Anabaena azolae, Azolla microphylla, Azolla pinnata, antimicrobe, rice crop

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1. INTRODUCTION

Rice (*Oryza sativa L.*) is the main food commodity in Indonesia because the staple food for most of Indonesia population is rice. The blast disease caused by *Pyriculari aoryzae Cav.* fungus and leaf blight disease caused by *Xanthomonas oryzae* bacterium are important diseases that attack paddy crop. These diseases strike during wet season or humid dry season, especially on constantly flooding paddy field.

Azolla is water fern plant that frequently found in paddy field area and it symbiosis with *Anabaena azollae* that capable to fix N_2 and subsequently convert N_2 into nitrogen available for crop (Muniappan et al., 2016). In addition to contribute nitrogen, *A. azollae* also produce plant growth hormone and antimicrobia compound (Renuka et al., 2018).

Sianobakter is a source of several compound having antimicrobial activity such as alkaloid, aromatic compound, depipeptide cyclic, peptide cyclic, undecapeptides cyclic, siclopane, extracellulair pigment, lipid acid, linear peptide, lipopeptide, nucleoside, phenol, macrolide, polyketides, polyphenyl ethers, porfinoide and terpenoid so that its extract can be used as antimicrobia (Swain et al., 2017; Salman and M.M, 2016). These bioactive compounds can inhibit the growth of soil induced pathogen bacteria such as *X. oryzae* bacterium (Abraham et al., 2015) and fungi such as *P. oryzae* that cause blast disease on rice crop (Blahova et al., 2013).

This research will conduct test in term of antibacterial and antifungal activities from compound produced by *A. azollae* which symbiosis with *Azolla sp.* on *X. oryzae* and *P. oryzae* that frequently cause disease on rice crop. It is expected that results of this research will provide an alternative biological control for blast and leaf blight diseases on rice crop by using *Azolla sp.* as well as N_2 contributor for crop.

2. EXPERIMENTAL SECTION

This research was conducted from November 2017 to April 2018. Sampling of Azolla which symbiosis with *A. azollae* was taken from Azolla cultivation pond at Faculty of Agriculture, Sriwijaya University. There are two types of Azolla, i.e. *Azolla pinnata* and *Azolla mycrophiylla*. Extraction of *A. azollae* from leaves of *Azolla pinnata* and *Azolla mycrophiylla* was done by using Ultrasound Asisted Extraction (UAE) with ethyl ester solvent and maseration method with methanol solvent. Rejuvenation of *X. oryzae* bacterium and *P. oryzae* fungus was done by using tilt agar medium. Analysis of active compounds from

extraction yield was done by using HPLC.

2.1 Extraction of *Anabaena azollae*

Azolla that had been air dried subsequently is blended to produce powder. In maseration method, 300 mL of methanol solvent is added into erlenmeyer containing 50 g dry biomass of azolla. This mixture is incubated for 7 days until the solvent's color change into dark green. After 7 days, supernatan is collected and dried by using Rotatory evapourator. In Ultrasound Asisted Extraction method, 40 mL ethyl acetate solvent is added into erlenmeyer containing 32 g dry biomass of azolla. This mixture is sonicated for 5 minutes, then it is shaked for 15 minutes by using shaker and subsequently it is centrifused at speed of 4000 rpm for 10 minutes. Supernatan is separated and collected within erlenmeyer. Biomass within pellete is added again with 40 ml ethyl acetate solvent. The extraction steps are repeated several times until all metabolites in biomass had been extracted which indicated by the change of cell biomass color into white color. The collected supernatan is subsequently dried by using Rotatory evaporator.

2.2 Preparation of Bacteria and Fungi

Pure isolates of *X. oryzae* bacterium and *P. oryzae* fungus are cultured on tilt agar media by taking colonies from stock of bacterium or fungus by using ose needle and it was scratched on tilt agar media. Tilt agar media is incubated at 37°C for 24 hours. Subsequently, colonies of bacterium or fungus from tilt agar media are taken by using sterile ose needle and each colony is put into test tube containing 10 mL Nutient Broth (NB) media.

2.3 Preparation of Test Media

Test media for bacterium used *Mueller Hilton* Agar (MHA) media with composition as follows: 6% peptone, 17.5% casein, 1.5% starch and 10% agar. Twenty five grams (25g) of MHA media is dissolved in 1000 mL aquadest and sterilized using autoclave at 121°C for 15 minutes.

Test media for fungus used *Sabouraud Dextrose* Agar (SDA) media with composition as follows: 10 g mycological peptone, 40 g glucose 40 g and 15 g agar. SDA media with magnitude of 6.5 g is dissolved with 100 mL aquadest within erlenmeyer and subsequently put on hot plate stirrer to homogenize the solution in order to produce clear solution. Media is sterilized in autoclave at temperature of 121°C for 20 minutes.

2.4 Test of Antibacterial and Antifungal Activities

Test of antibacterial and antifungal capabilities was done by using disk paper diffusion method. Sterile media of MHA and SDA are respectively poured into petri dish with thickness of ± 0,5 cm and waited until condense at room's temperature. Suspension of bacterium and fungus respectively with magnitude of 0.1 mL are innoculated using spread plate technique in surface of MHA media for bacterium and in surface of SDA media for fungus. Disk from sterile filter paper with diameter of 5 mm is saturated with ethyl acetate or methanol extract of A. azollae which life within leaves of *A. pinnata* and *A. mycrophylla*. Filter paper is put in center of agar surface that had been innoculated with bacterium or fungus, then it is covered and put in upside down position within incubator at temperature of 37°C for 24 hours. Diameter of produced clear zone is an indication of extract's inhibitory power toward bacterium or fungus. Inhibitory zone diameter is measured by using vernier caliper. The different of produced inhibitory zone diameter are compared by using t-test. Analysis of active compounds from ectraction yield was done by using HPLC.

3. RESULTS AND DISCUSSION

3.1 Test of Antibacterial and Antifungal Activities

Figure 1 showed the development of growth inhibitory zone for bacterium or fungus by antimicrobial compounds from ethyl acetate or methanol extract of *A. azollae* which life within leaves of *A. pinnata* and *A. mycrophylla*.

An extract has potential as antimicrobia if it produces microbial growth inhibitory zone during the test. The magnitude of produced inhibitory zone is affected by antimicrobal compound activity on this extract. Salman and M.M (2016) showed that *A. azollae* can produce toxic compound as antibacterial agents such as alkaloid, neurotoxins and anatoxin. Rossana et al. (2006) had reported that antifungal compound produced by A. azollae which life in leave tissue of *Azolla sp.* are phenol, flavonoid, alkaloid, terpenoid, glycoside and saponin. Alkaloid compound has inhibitory mechanisms by disturbing peptidoglycan constituent components within cells so that cell wall layers are not fully developed resulting in death of cells (Juliantina, 2008). Gunawan (2009) had stated that base group containing nitrogen which is available within alkaloid compound reacts with amino acids that compile cell wall and DNA of bacterium and fungus. This reaction results in the change of structure and arrangement of amino acids which in turn produce the change of genetic equilibrium in DNA chains and damage which promote lysis and death of cells on bacterium.

The capability differences of ethyl acetate or methanol extract of *A. azollae* from leaves of A. microphylla and A. pinnata in inhibiting the growth of *X. oryzae* bacterium and *P. oryzae* fungus is shown in Table 1.

Tabel 1 showed capability effect of *A. azolae* extract from leaves of A. microphylla and *A. pinnata* on activitities of *X. orizae* bacterium and *P. oryzae* fungus. There was significant different between inhibitory zone diameter of bacterial activity by *A. azollae* extract from leaves of *A. microphylla* and *A. pinnata* using the same solvent, either ethyl acetate or methanol. However, there was no significant different between inhibitory zone diameter of *P. oryzae* fungus activity by *A. azollae* extract from leaves of *A. microphylla* and *A. pinnata* using the same solvent, either ethyl acetate or methanol.

Tabel 2 showed test results of capability differences of *A. azollae* extract from leaves of A. microphylla and A. pinnata using different solvents toward *X. oryzae* bacterium and *P. oryzae* fungus activities. There was significant different between inhibitory zone diameter toward *X. oryzae* bacterium and *P. oryzae*

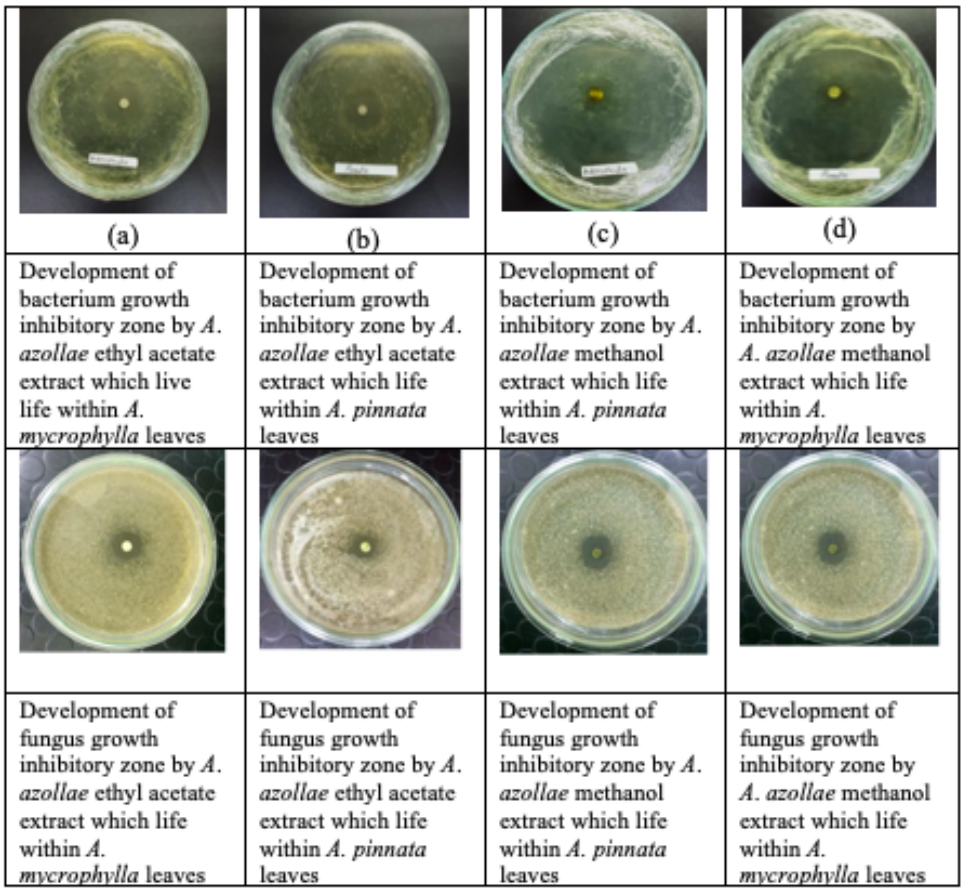


Figure 1. Development of growth inhibitory zone for *X. oryzae* bacterium or *P. oryzae* fungus on agar media

Table 1. Capability difference of *A. azolae* extract from *A. microphylla* and *A. pinnata* leaves with the same solvent

Extraction source of *A. azollae*	Solvent	Inhibitory zone diameter (mm)	t-calculated	t-table 0.05
On activity of *X.oryzae* bacterium				
Azolla microphylla	Ethyl acetate	13	3.45*	2.13
Azolla pinnata	Ethyl acetate	15		
Azolla microphylla	Methanol	10	2.59*	2.13
Azolla pinnata	Methanol	11.5		
On activity of *P. oryzae* fungus				
Azolla microphylla	Ethyl acetate	16.25	0 46^{tn}	2.78
Azolla pinnata	Ethyl acetate	15.58		
Azolla microphylla	Methanol	22.25	2 35^{tn}	2.78
Azolla pinnata	Methanol	21.33		

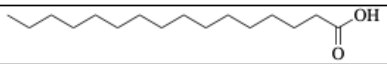
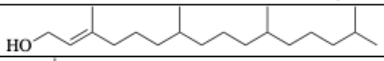
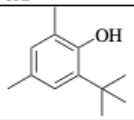
No	Chemical structure	Name of chemical compound
1.		Hexadecanoic acid
2.		Phytol
3.		2-(tert-butyl)-4,6-dimethyl phenol

Figure 2. Chemical structures for three compounds within *A. azollae* extracted from *A. pinnata* and *A. microphylla* leaves

fungus activities due to treatment of *A. azollae* extract from leaves of *A. microphylla* and *A. pinnata* using different solvent.

Table 2 showed that four *A. azollae* extracts had capability in inhibiting the growth of bacterium and fungus. This in accordance to the study results by Nabakishore et al. (2015) which showed that extract from *A. azollae* can inhibit the growth of Staphylococcus aureus. *A. azollae* extract with methanol solvent had lower antibacterial activity than that of using ethyl acetate solvent. This in accordance to the study results by Moshi and Moshi and Mbwambo (2005) which showed that semi polar extract (ethyl acetate) can inhibit E. coli and B. anthracic bacteria with higher inhibitory diameter than that of polar extract (methanol). On the other hand, antifungal activity from extract with methanol solvent was higher than that of using ethyl acetate solvent. Methanol that has high polarity is capable to dissolve most of compounds having antifungal property. Solvent with low polarity such as ethyl acetate draws antifungal active extract in less quantity than that of ethanol and methanol mixture or methanol alone (Ismail et al., 2004).

3.2 The compounds available in *A. azolae* extract

Factors which cause differences in pathogen inhibitory power are variation and concentration of secondary metabolites contains within extract and the produced fraction. Table 3 showed compounds available in *A. azolae* extract which life within leaves of *A. pinnata* and *A. mycrophylla* using ethyl acetate and methanol solvents. Widiana (2012) had reported that one of factors that affect antimicrobia in inhibiting microbia growth is concentration of antimicrobial substance. The higher the concentration of extract containing antibacteria, the faster the killing process of pathogen. Microorganisms have different tenacity to antimicrobia. The higher the number of microbial cells, the longer the treatment time required to kill all microbia.

Table 3 showed that dominant compounds within *A. azollae* extract with probability higher than 60 % and area of more than 10 % are consisted of phytol, hexadecanoic acid and 2-(tert-butyl)-4,6-dimethyl phenol. Figure 2 showed that chemical structures for three compounds in Table 3.

In accordance to the published papers by Rossana et al. (2006); Salman and M.M (2016); Swain et al. (2017), the above compounds (hexadecanoat acid, phytol, and phenol) had antimcrobial activity. Further research is needed to determine the possibility of compounds that contains in A. azollae extract can be applied as biocide.

4. CONCLUSIONS

Ethyl acetate or methanol extract of *A. azollae* which symbiosis with *A. pinnata* or *A. microphylla* was capable to inhibit the growth of *X. oryzae* bacterium and *P. oryzae* fungus. The dominant compounds containing in extract of *A. azollae* with probability higher than 60 % and area of more than 10 % are consisted of phytol, hexadecanoat acid and 2-(tert-butyl)-4,6-dimethyl phenol.

5. ACKNOWLEDGEMENT

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Table 2. Test of capability differences of *A. azolae* extraction from *A. microphylla* and *A. pinnata* leaves on activity of *X. oryzae* bacterium

Source of *A. azollae* extraction	Solvent	Inhibitory zone diameter (mm)	t-calculated	t-table 0.05
On activity of *X.oryzae* bacterium				
Azolla microphylla	Ethyl acetate	13	4.23*	2.13
Azolla microphylla	Methanol	10		
Azolla pinnata	Ethyl acetate	15	5.12*	2.13
Azolla pinnata	Methanol	11.5		
On activity of *P. oryzae* fungus				
Azolla microphylla	Ethyl acetate	16.25	5.39*	2.78
Azolla microphylla	Methanol	22.25		
Azolla pinnata	Ethyl acetate	15.58	5.76*	2.78
Azolla pinnata	Methanol	21.33		

Table 3. Probability, area and retention time of compound formed from *A. azollae* extracted from *A. pinnata* and *A. microphylla* leaves

Extraction sources	Compound formed	Probability (%)	Area (%)	Retention time (minutes)
A. pinnata with ethyl acetate solvent	Phytol	74.09	15.88	19.56
	Hexadecanoic acid	68.27	16.01	18.74
A.microphylla with ethyl acetate solvent	Phytol	73.68	11.89	19.55
	Hexadecanoic acid	77.57	15.29	18.26
A. pinnata with methanol solvent	2-(tert-butyl)-4,6-dimethyl phenol	95.81	17.05	22.1
	Hexadecanoic acid	76.23	12.67	18.25
A. microphylla with methanol solvent	2-(tert-butyl)-4,6-dimethyl phenol	69.69	10.62	19.55
	Hexadecanoic acid	60.22	14.84	18.5

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