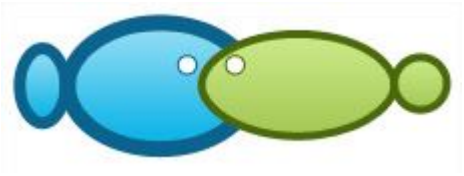

In vitro test of natural antibacterial activity of yellow-fruit moonseed *Arcangelisia flava* Merr. leaf on bacterium *Pseudomonas fluorescens* under different doses

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Abstract. *In vitro* study on antibacterial activity of yellow fruit moonseed *Arcangelisia flava* Merr. leaf on bacteria *Pseudomonas fluorescens* was done to deal with bacterial infection in freshwater culture. This study was intended to find alternative source of antibacterials from nature, in this regard, yellow fruit moonseed *A. flava* leaf. It is expected to be able to inhibit the growth of *P. fluorescens* that causes freshwater fish mortality and not to result in resistance. The phytochemical test indicated that *A. flava* leaf was detected containing secondary metabolic compound, alkaloid, phenol, flavonoid, and saponin. All active compounds of *A. flava* leaf enable it to be developed as natural antibacterials due to being capable of inhibiting bacterial growth. This study used diffusion method through disc paper. Results showed that *A. flava* leaf possessed inhibition activity on the growth of *P. fluorescens*, through inhibition zone measurements around the paper disc, and thus, the size of the inhibition zone could be determined. The minimum inhibition concentration of *A. flava* on *P. fluorescens* was 1.5% with mean inhibition zone of 7.18 mm.

Key Words: minimum inhibition concentration, phytochemical test, active compound, bacterial growth.

Introduction. The largest constraint in freshwater culture is disease infection that can cause quality decline of the aquaculture production from fish mortality and harvest failure. Pseudomonas belongs to dangerous disease agents in freshwater fish. It also could cause high fish mortality since this disease could disperse very fast if the water condition is possible (Hasan et al 2013; Nurjanah et al 2014).

Infection of bacteria *Pseudomonas fluorescens* in fish can make ulcers on fish skin, fins, abdominal cavity, and internal organs. This bacterium can also cause anemia and mass mortality (Kordi & Ghufran 2004; Darak & Barde 2015; Saharan et al 2015).

Disease treatment on fish infected with *P. fluorescens* is conducted using antibiotics, but the use of antibiotics could, in fact, result in side effects on the pathogens or the cultured fish. Continuous antibiotic application could make the pathogen be resistant that causes the use of antimicrobes be ineffective. Besides, the residue of the antibiotic could pollute the aquatic environment (Retnawati 2008; Tiwari et al 2013).

Dealing with the problems, safer alternative medicinal materias are needed in fish disease prevention. One of the alternatives is the use of traditional medicinal plants holding antibacterials. Several benefits of this usage are relatively safer, easily obtained, cheap, causing no resistance, and relatively not hazardous for surrounding environment (Sugianti 2005; Singh 2015; Hosseinzadeh et al 2015). This study is expected to be able to find alternative medicines from antibacterial-containing-natural chemicals of *Arcangelisia flava* Merr. leaf capable of inhibiting the growth of *P. fluorescens* that could cause freshwater fish mortality

A. flava leaf has been known and utilized by peole of Central Kalimantan, particularly Dayak communities as traditional medicine, since it has been able to cure various infection diseases, one of which is hepatitis. Phytochemical tests indicated that

the root of *A. flava* was proved to have secondary metabolite compounds, such as alkaloid, saponin, terpenoid, and flavonoid (Maryani et al 2013). Leaf is thought to hold antibacterial compounds useful for bacterial infection treatment. Study on A. *flava* leaf has not been done a lot, and therefore, its potential as alternative antibacterial from natural material needs to be studied to treat the bacterial disease that is safe for cultured fish, environment, and human in long-term application.

The objective of the study is to know the compound content, antibacterial activity, the best concentration of *A. flava* leaf in inhibiting in vitro growth of *P. fluorescens*. This study is expected to be able to provide information on the potential of *A. flava* leaf slow down the growth of *P. fluorescens.*

Material and Method. This study was carried out in Fish Health Laboratory of Fisheries Department, Pangkaraya University, Central Kalimantan, Indonesia, from October 2016 to April 2017. Materials used were bacteria *P. fluorescens* ATCC 49642, *A. flava* leaf, Tripticase Soy Agar (TSA) produced by BBL (BD Diagnostic Systems), standard solution Mc Farland, methanol, ethanol, aquadest, phytochemical screening reagents (Mayer, Wagner and Dragendorff, gelatine salt 2%, ammonia 25%, Na_2SO_4, HCl 2M, $FeCl_3$ 0.1%, H_2SO_4 10%, NaOH 1 M, NaCl 10%, and $CHCl_3$), filter, filter paper, labeling sheet, aluminium foil, and paper disk. The equipments were blender (Phillip), Erlenmeyer (Pyrex), rotary evaporator (N-1001S-W, EYELA-USA), digital waterbath (SB-1000 EYELA-USA), petri disk, micropippet, shelf, flask, funnel, ose needle, freeze dryer, laminar flow, HL-36AE Hirayama-typed autoclave, bunsen burner, digital caliper series 500, glass bottle, and vortex. The study used experimental method.

Sample processing. *A. flava* leaf was washed, drained, chopped, and dried in the oven at 60^{o}C, sorted the dry simplicia and milled into powder using blender, packed and stored. The leaf sample was kept in a dry plastic bag and stored at room temperature.

Simplicia powder extraction. The simplicia was macerated in 5 liters of 96% methanol solvent for 5x24 hours. The macerate was then filtered and evaporated using vacuum rotary evaporator at 55^{o}C. The extract was reevaporated in a water bath to obtain viscous extract, then kept in the glass container layered with aluminium foil.

Phytochemical screening. Phytochemical examination was done to check the presence of alkaloid, phenol, tanin, flavonoid, and saponin.

Alkaloid. The presence of alkaloid was determined using Mayer, Wagner, and Dragendorff method (Kayaputri et al 2014). Three mL of sample were put in a porcelain cup, added with 5 mL of 2M HCl, stirred, and cooled at room temperature. The cool sample was added with 0.5 g of NaCl, stirred, and filtered. The filtrate was added with 3 drops of 2M HCl, and then divided into 4 portions, A, B, C, and D, respectively. Filtrate A was blank, B was added with Mayer reagent, C was added with Wagner reagent, and D was used as assertion test. Dreg formation at the addition of Mayer and Wagner reagents indicated the presence of alkaloid. The assertion test was done by adding 25% ammonia to filtrate D up to pH 8-9, then added with chloroform, and evaporated on the waterbath. It was then added with 2M HCl, stirred, and filtered. The filtrate was divided into 2 parts, A was blank, B was tested with Mayer reagent, and C was tested with Dragendorff reagent. Dreg formation indicated the presence of alkaloid.

Phenol. Phenol presence was determined following Solomon-Wisdom et al (2014). Two mL of sample (±0.05% b/v) was dissolved in 10 mL aquadest, heated for 5 minutes, and filtered. The filtrate was added with 4-5 drops of 5% $FeCl_3$ (b/v). Phenol presence was indicated with the formation of dark blue color or blackish green.

Tanin. This compound was detected following Kayaputri et al (2014). As much as 3 mL of sample were extracted with hot aquadest, and then cooled. It was then added with 5 drops of 10% NaCl and filtered. The filtrate was divided into three parts, A, B, and C, in

which A was used as blank, B was added with 3 drops of $FeCl_3$, and C was added with gelatin salt, then observed the change.

Saponin. Saponin test employed Forth method (Kayaputri et al 2014) by inserting 2 mL of sample into the flask, then added with 10 mL of aquadest, stirred for 30 seconds, and observed the change. When the foam is formed (not lost for 30 seconds) then shows the presence of saponins. The assertion test of saponins was done by vaporizing the sample to dry and then washed off with hexane until the filtrate was clear. The residue was added with chloroform, stirred for 5 minutes, then added with anhydrous Na_2SO_4, and filetered. The filtrate was separated into 2 parts, A and B, respectively, in which A was used as blank, and B was dropped with anhydrous acetate, slowly stirred, then added with concentrated H_2SO_4 and restirred. The formation of red to brown ring indicates the presence of saponin.

Flavonoid. This compound was detected using Bate Smith-Metchalf method and Wilstater method (Kayaputri et al 2014). As much as 3 mL of sample were evaporated, and washed with hexane up to clear. The residue was dissolved in 20 mL of ethanol, then filtered. The filtrate was divided into 3 parts, A, B, and C, respectively, in which A was used as blank, B added with 0.5 mL of concentrated HCl then heated at the water pan. Change to red to purple color indicates the presence of flavonoid (Bate Smith-Metchalf method). Filtrate C was added with 0.5 mL HCl and Mg, then observed the color change (Wilstater method). Red to orange color was produced by flavon compound, dark red was given by flavonols or flavonone, and green to blue is given by aglycone or glycoside.

Preparation of testing solution of A. flava leaf. Testing extract was prepared in different concentrations: 3.5%, 3%, 2.5%, 2%, 1.5%, 1%, and 0.5%.

Antibacterial activity test using diffusion method. This testing was conducted on Tripticase Soy Agar (TSA). Each agar plate contained 15-30 mL of liquid agar that was then compacted. The surface of TSA media was inoculated with test bacteria by greasing bacterial suspension-containing cotton on the media surface. The disc placed on the TSA plate must have minimum distance of 24 mm from each disc center. Each disc was poured 20 µL of different test concentration. After all processes had finished, the petri discs were inserted into an incubator at 37^{o}C for 24 hours. The transparent area around the disc indicates no bacterial growth and interpreted as inhibition zone (Brooks et al 2013; Balouiri et al 2016).

Observed parameters. The inhibition zone was measured using a caliper and interpreted as the strength of the inhibition zone. The inhibition zone formed in the lowest concentration of *A. flava* leaf extract was taken as minimum inhibition concentration.

Results and Discussion

Phytochemical screening. Identification of secondary metabolite content is an important initial step in searching new bioactive compounds of natural materials that can become precursor of new drug synthesis or certain active drug prototype (Harborne 2006; Solomon-Wisdom et al 2014; Sahira & Cathrine 2015). The secondary metabolite group identified from *A. flava* leaf extract is presented in Table 1.

Based on phytochemical tests, *A. flava* leaf extract was detected containing several secondary metabolite compounds, alkaloid, phenol, flavonoid and saponin. The presence of these secondary metabolite compounds makes *A. flava* leaf enable to be developed as natural antibacterial.

Table 1

Phytochemical screening on *A. flava* leaf extract

Phytochemical test	*Reagents*	*Color change*	*Remark*
Alkaloid	Mayer	White precipitate	+
	Wagner	Brown precipitate	+
Phenol	$FeCl_3$ 3%	Blackish green	+
Tanin	$FeCl_3$ 1%	Blackish green	-
	Gelatin 2%	White precipitate	-
Flavonoid	Mg + concentrated HCl	Yellow	+
	Zn + 2N HCl	Sensitive red	+
Saponin	Hot aquadest	1 cm foam for 30 second	+

+: present; -: not present.

The secondary metabolite compound, alkaloid, according to Robinson (1995) and Mabhiza et al (2016), possesses antibacterial activity by disturbing the formation of peptidoglycan cross bridge of the bacterial cells so that the cell wall layer is properly formed, and result in cell lysis. The cell wall damage mechanism could result from accumulation of lipophilic components in the cell wall or cell membrane which alters the cell wall building composition (Drosinos et al 2000; Van Wyk & Wink 2004). In such a situation, the bacteria will not grow and even could die.

Phenolic group could damage the cell membrane, activate the enzyme, and denaturate protein so that the cell wall is damaged from permeability decline. Change in the permeability of cytoplasmic membrane could disturb transportation of important organic ions into the cell that inhibits growth or even kills the cell (Damayanti & Suparjana 2007). In high concentration, phenols can penetrate and disturb the cell wall of bacteria, and precipitate protein in bacteria cells (Oliver et al 2001; Salem et al 2013).

The antibacterial mechanism of flavonoid is to disturb peptidoglycan transpeptidase activity so that the cell wall formation is disturbed and the cell experiences lysis (Cowan 1999; Kim et al 1995), while saponin is an active substance that can increase membrane permeability so that if saponin interacts with bacteria cells cell hemolysis will occur, and the bacteria will be damaged (Poeloengan & Praptiwi 2010; Madduluri et al 2013).

Minimum inhibition concentration test using diffusion method. Minimum inhibition concentration can be seen on the extent of the inhibition zone formed around the paper disc. Table 2 presents the inhibition zone diameter data of *P. fluorescens.*

Table 2

Inhibition zone diameter of *A. flava* leaf extract against *P. fluorescens*

Concentration of testing solution (%)	*Diameter of inhibition zone (mm)*			*Mean*	*Standard deviation*	*Extract strength*
	I	*II*	*II*			
3.5	14.23	13.76	14.53	14.17	0.39	Moderate activity
3	13.67	13.06	13.42	13.38	0.31	Moderate activity
2.5	9.69	10.02	9.55	10.34	0.33	Weak activity
2	8.56	7.86	8.26	8.23	0.55	Weak activity
1.5	7.55	6.83	7.15	7.18	0.36	Weak activity
1	0.00	0.00	0.00	0.00	0.00	No activity
0.5	0.00	0.00	0.00	0.00	0.00	No activity

The concentration of 0.5-1% does not show any inhibition zone. The smallest mean inhibition zone diameter (minimum inhibition zone) occurs at the concentration of 1.5%, 7.18 mm, and the largest mean inhibition zone occurs at the concentration of 3.5%, 14.7 mm. The antibacterial activity is affected by several factors, i.e. extract concentration, antibacterial compound content, extract diffusibility, inhibited bacterial species (Brooks et al 2013; Ali-Shtayeh et al 1998; Ramkumar et al 2007). The present study found that the higher the concentration of *A. flava* leaf extract is, the larger the inhibition zone

against *P. fluorescens,* so that it could be assumed that the higher the extract concentration is, the higher the number of antibacterial compounds is released, and the penetration of the compound into the bacterial cell is easier through its own mechanisms.

Based on the extract strength classification in inhibiting the growth of *P. fluorescens*, it was found that *A. flava* leaf extract concentration of 1.5-2.5% had weak activity and the concentration of 3-3.5% had moderate activity.

Present study found difference in bacterial growth because due to different concentrations. This condition is similar to that reported by Pelczar & Chan (1988) that antibacterial activity is affected by the substance concentration. Three and a half percents are the concentration giving the largest inhibition zone categorized as moderate activity. The extract concentration of 3.5% yielded the largest inhibition zone with moderate activity, and thus, it was the effective concentration among all tested concentration variations. It is also in line with Faozi (2013) that the higher the extract concentration is, the bigger the inhibition zone of bacterial growth caused by the active compound of *A. flava* leaf will be. Boyd (1995) also stated that the lower the concentration is the lower the ability of the antibacterial substances to inhibit the bacterial growth.

A. flava leaf could be effective to use as medicine, because in low concentration it is capable of inhibiting bacteria *P. fluorescens in vitro*. Ernst (1991) stated that drug treatment is beneficial if it is administered in sufficient concentration and not excessive. This study found that application of 1.5 g mL^{-1} was enough to inhibit bacterial growth.

Conclusions. Phytochemical test revealed that *A. flava* leaf extract contained secondary metabolite compounds, alkaloid, phenol, flavonoid, and saponin. It also reflected antibacterial potential against *P. fluorescens* indicated with the formed transparent zone. The highest inhibitability was found at the extract dose of 3.5% mean inhibition zone diameter of 14.17 mm, and the minimum inhibitability was recorded at the concentration of 1.5% with mean inhibition zone of 7.18 mm. In vivo study was also needed to control the bacterial infection of *P. fluorescens* in freshwater aquaculture.

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