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In Vitro Potential Test of Ketapang (*Terminalia catappa*) Leave Extract against *Aeromonas salmonicida*

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ABSTRACT

Ketapang leave had known potential well as bactericide toward few bacteria. This research was aim to measure the zone of inhibition which formed in every concentration of ketapang (*Terminalia catappa*) extract, minimum extract concentration of T. *Catappa* leave to potentially detain and eradicate A. *Salmonicida*. Clear zone, both number of *Minimum Inhibitory Concentration* (MIC) and *Minimum Bactericidal Concentration* (MBC) were put as observation object. Zone of inhibition diameter at disk diffusion test analyzed statistically with ANOVA but number of bacterial coloni within tube dilution test analyzed descriptively. Diffusion test result with vary concentration treatment of it gave significantly different toward clear zone formation. The best value obtained at concentrations 400 mg / ml, with a value of the average diameter clear zone around 23.167 ± 0.764. The MIC value around 440 mg/ ml had obtained in test tube dilution, while the MBC value was 480 mg/ ml.
KEYWORDS—Aeromonas salmonicida, antibakteri, ekstrak daun, Terminalia catappa.

INTRODUCTION

Indonesia was a tropical country that had enough medicinal plant inventory overflows [1]. One of them, namely ketapang (*Terminalia catappa*), leaves and fruit of it reportedly had a content of anticancer, antioxidant, anti-HIV, and antidiabetic. Young plant leaf extract of it used as an external treatment in the form of an ointment to treat leprosy and scabies, and for internal medicine to cure a headache [2].

Ketapang naturally grew on sandy beaches or stony. It was able to tolarete of land masin, impervious to sea water splashing and hardly resistant to the wind. Into all kinds of soil with good drainage, on low-lying parts of the coast until the highlands of with a height of 800 m above ocean survaces with sufficient rain precipitation these plants grew well. It was belong to southeastern Asia native herbs, which could be found almost across in Indonesia however on the Borneo these plants rarely found [3].

T. *catappa* was potentially as an antibacterial because containing secondary metabolite compounds that were a tannin, flavonoid and saponin [4]. Based on the antibacterial experiment used it leaves at different stages (young, immature, and old leaves shall drop) stated that in the adult stage had the highest inhibition by the diameter of 17.2 mm against bacteria *Pseudomonas aeruginosa*, at the young stage only 12.4 mm diameter, and 6 mm at the hoary leaves stage [5]. According MIC (Minimum Inhibitory Concentration) test, amount 0.5 mg/ ml of it extract had known able to against bacteria *Aeromonas hidrophila* [6].

A metabolite secondary compound was applicably role as an antibacterial agent in fishery sector (treatment for disease caused by the bacterium). *Aeromonas salmonicida* was one of a disease-causing bacterium at the fish [7]. Bacteria were major causes of disease furunculosis and carp erytrodermatitis [8].

This research was aim to measure the zone of inhibition which formed in every concentration of ketapang (*Terminalia catappa*) extract, minimum extract concentration of T. *Catappa* leave to potentially detain and eradicate A. *Salmonicida.* In vitro research is required to know the antibacterial effect of *Terminalia catappa* extract leaves for inhibiting the growth of *Aeromonas salmonicida* bacteria. It information result placed as bridge for upcoming in vivo research.

METHODS

Composing antibacterial substance

Ketapang leaves cleared from impurities, washed by water to clean, cut into pieces, drained, dried, then it blended till to be powder. It extracts leaves as many as 250 gr incorporated into a glass jar that contains 1 L methanol. Glass jar closed and left for 24 hours. It extract concentrated in a Rotary vacuum evaporators under 40^0 C temperature [9]. It diluted with DMSO 10% (Dimethil Sulfoxide) in accordance with the concentration which expected [10]. Ampisillin utilized as positive control while DMSO 10% set as negative control.

Culture of bacteria

Aeromonas salmonicida were Primary Bereau for Fish Quarantine (Balai Karantina Ikan Kelas I) Juanda, Sidoarjo collection. They inoculated at Tryptic Soy Agar (TSA) by strek plate method after 24 hours incubation in room temperature.

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Their colonies were subculture using the inoculating loop at Tryptic Soy Borth (TSB) and incubated again as long as 24 hours. *Aeromonas salmonicida* tumbuh optimally grew under 20^0C- 30^0C while at 35^0C got slow down till death at 37^0C [11].

Disk diffusion test

Extract made into 8 concentrations (50, 100, 150, 200, 250, 300, 350, 400 mg/ ml) with 3 times for replications. Disk Diffusion Test conducted to obsereve the zone of inhibition formation over all concentrations. It played for testing bacteriside capability where Ampisillin utilized as positive control while DMSO 10% set as negative control. As many as 0.1 ml *Aeromonas salmonicida* suspension (0.5 Mc. Farland standart) inoculated using spread plate method at TSA solid medium. Paper disk dried after dipped in each concentration, positive and negative control. It placed in TSA plate medium. Plate incubated as long as 24 hours [12]. Clear zone measured surround the disk to show zone of inhibition against bacteria.

Tube dilution test

Tube dilution test used to determine both MIC and MBC values. Determining for MIC (Minimum Inhibitory Concentration) acted by added 4.5 ml of TSB medium + 0.5 ml extract + 0.25 ml *Aeromonas salmonicida* suspension (0.5 Mc. Farland standart) into 8 concentrations in each test tube. It homogenized then incubated as long as 24 hours [14]. The results of observation compared to standart solution (TSB medium added extract without bacterial suspension). Medium who began to clear, it performed MIC value. MIC values defined as the highest dilution and the lowest concentration of samples [15].

MBC determined after inoculated solution from clear MIC tubes at medium [16]. 0.1 ml bacterial suspension grew and incubated as long as 24 hours at TSA medium where it taken from test tube who determine MIC value. Bacterial colonies, which growing at TSA medium, are calculated. MBC value reached from the lowest extract concentrations that no colonies could be seen at the Petri disk [14].

Research scheme and Data analysis

Completely Randomized Design with 10 treatments and 3 times replications used at Disk Diffusion Test. Data were gain from celar region measurment. They were either analyzed in descriptive qualitative and using *Analysis of Varian* (ANOVA) one way with 95% level of convidence. If significant differ occured continued by Tukey test.

Research sheme and data analysis for Tube Dillution Test derived from11 treatments. MIC (Minimum Inhibitory Concentration) test result observed by comparing to control where data descriptively analyzed. Whereas MBC (Minimum Bactericidal Inhibitory) test result, bacterial colonies calculated by colony counter and also descriptively analyzed.

RESULTS AND DISCUSSION

Antibacterial test using Disk Diffusion Test method

Ketapang (T. *catappa*) leaves extract proved potentially as antibacterial against A. *salmonicida.* It supported by zone of inhibition formation at metode disk diffusion test method in each concentration. P-value 0.000 ($P < 0.05$) of ANOVA test was show that it impacted to hamper bacterial growth. Granting of 400 mg/ml concentration of Ketapang (T. *catappa*) leaves extract carried weight significantly to inhibit A. *salmonicida* growth by forming clear zone diameter as much 23.2 mm (Figure 1).

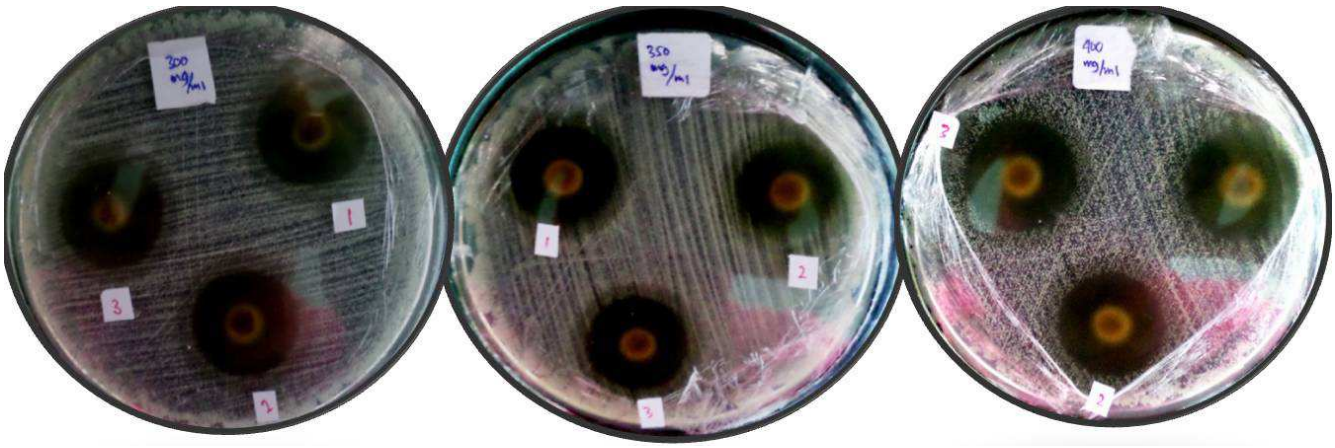

Figure 1. Clear zone formation of *Terminalia catappa* leaves extract against A. *salmonicida* using disk diffusion test at concentration of (a) 400 mg/ml, (b) 350 mg/ml, and (c) 300 mg/ml.

The results of Tukey test gave significant difference in the treatment group by the addition of a ketapang (T. *catappa*) leaves extract on the concentration of 400 mg / ml against bacteria A. *salmonicida*, after compared with the treatment group other. Bottleneck classifications response based on a Greenwood, Table 1[13].The average score followed by the different letters had a

significant difference (α 0.05) were the outcome of the diffusion. Based on ANOVA test can be seen that in the concentration of 350 mg/ ml markedly dissimilar not by concentration of the 300 mg/ ml (Table 2). In the concentration of 50 mg/ mls, 100 mg/ ml, 150 mg/ ml, 200 mg/ ml, and 250 mg/ ml gave medium response, shown with the establishment of clear zone each of them; at 16.4 mm 17.2 mm; 17.9 mm; 19.5 mm; 19.75 mm. Clear zone diameter formed with range of 16-20 mm were classified as medium response [13].

Table 1. Bottleneck Responses clasification [13]

Zone of Inhibition Diameter	Bottleneck Responses of Growth
≤ 10 mm	None
11-15 mm	Lemah
16-20 mm	Sedang
>20 mm	Kuat

Table 2. The averages for zone of inhibition diameter by Ketapang extract leaves against *Aeromonas salmonicida*

Extract concentration	The averages for zone of inhibition diameter (mm)	Bottleneck Responses
Kontrol negatif	0,000±0.000^{f}	None
Kontrol positif	17,000±0.250^{e}	Medium
50 mg/ml	16,417±0.520 ^{e}	Medium
100 mg/ml	17,167±0.520 ^{e}	Medium
150 mg/ml	17,917±0,629^{de}	Medium
200 mg/ml	19,500±0,500^{cd}	Medium
250 mg/ml	19,750±0,901^{bc}	Medium
300 mg/ml	21,333±0,289^{b}	Medium
350 mg/ml	21,417±0,804^{b}	Strong
400 mg/ml	23,167±0,764^{a}	Strong

Positive control (ampisillin 50 mg/ml) shaped clear region around 17 mm, it gave medium responses (Table 2) to hamper A. *salmonicida* growth. Generic variant of ampicillin utilized at this research so that it allegedly made medium responses. It had widely bacteriostatic spectrum, persisted to acid, poor to penisilinase, and very active bactericide. Detaining wall cell formation by disputed conjoined N-asetilmuramat acid was it mechanism [17].

No significant difference presented in ANOVA test between ampisillin to Ketapang (T. *cataappa*) leaves extract at 50 mg/ml and 100 mg/ml concentrations. Negative control (DMSO 10%) did not give retardation to A. *salmonicida* bacterial growth it supported with the absent of clear region. That was appropriate with Sharma and Sharma, under 15% concentration of DMSO [18].

Ketapang (T. *catappa*) leaves extract at 50 mg/ml concentration had the lowest antibacteria activity which constructed 16.4 mm clear zone. Whereas 400 mg/ml concentration got the highest antibacteria activity with 23.2 mm formation zone of inhibition. As more granting of ketapang (T. *catappa*) leaves extract as much more zone of inhibition formation. semakin besar diameter zona bening yang terbentuk. This result also stated by Pelczar and Chan [19].

Zone of inhibition formed by T. *Catappa* leaves extract granting had shown that A. *Salmonicida* growth are inhibited due to secondary metabolite compound within it. Secondary metabolite was common chemical compound that had bioactive capability and played as plant protector from environment and pest. One of pest was bacteria where it acted also as antibacteria compound [20].

According to Neelavathi, T. *catappa* contained tannin, flavonoid and saponin within their leaves extract [21]. Thus Phytochemical test continued by Ultraviolet-Visible Spectrophotometry. The result informed that 8.21% of tannin, 4.72% of saponin, and 1.38% of flavonoid in it contains.

Tannins were most abundant among other compounds in Phytochemical test which were 8.21%. Together with Phospholipids within bacteria cell wall, they interacted so that it corrupted primary metabolite and inactivated bacterial enzymatic system. Devastating of cell wall prevented incoming materials and nutritions flow where those are required to gain energy. Finally bacteria had barriers to grow and even run down [22].

Saponin placed as the 2nd highest percentage (4.72 %.) after tannin compound. Saponin was a compound substance that increased membrane permeability so lead hæmolysis cell occured, if saponin interacted to bacteria it had resulted bacteria cell walls rupture or lysis [23].

In Phytochemical test, flavonoid compounds was the lowest percentage (1.38 %) compared to tannin and saponin. Flavonoids were phenol derivative compound. It damaged to the composition and changed the permealibity mechanism of bacterium cell wall so that called antibacterial [24].

Antibacterial test using Tube Dilution Test method

Tube dilution test was consist from 2 method which were MIC and MBC. MIC (Minimum Inhibitory Concentration*)* intended to get minimum concentration of *Terminalia catappa* leaves extract that potentially inhibited *Aeromonas salmonicida.* MBC (Minimum Bactericidal Concentration) was head to achieve minimum concentration of it to exterminate them.

Table 3. The MIC values of Ketapang extract leaves against *Aeromonas salmonicida*

Extract concentration	Determination of MIC values	∑ number of Bacteria colonies (CFU/ml)
0 mg/ml	Turbid	>>>
300 mg/ml	Turbid	0,00616x10^6
320 mg/ml	Turbid	0,00570 x10^6
340 mg/ml	Turbid	0,00562 x10^6
360 mg/ml	Turbid	0,00416 x10^6
380 mg/ml	Turbid	0,00373 x10^6
400 mg/ml	Turbid	0,00340 x10^6
420 mg/ml	Turbid	0,00277 x10^6
440 mg/ml	clear	0,00141 x10^6
460 mg/ml	clear	0,00070 x10^6
480 mg/ml	clear	0
500 mg/ml	clear	0

Mark >>> informed unable counted too much colonies.

Tube dilution test method utilized 0, 300, 320, 340, 360, 380, 400, 420, 440, 460, 480, and 500 mg/ml concentrations. On the MIC test outcome, in concentration 0 to 420 mg / ml cloudiness were present in TSB media that inoculated bacteria A. *salmonicida* after incubated for 24 hours, it proved in that media bacteria had arised. MIC value pointed at 440 mg / ml concentration where shown with a clear solution who began (Figure 2). It indicated that the growth of bacteria A. *salmonicida* inhibited by Ketapang leaves extract from 440 mg /ml to the concentration of 500 mg/ ml.

MBC value determined from the lowest concentration of extract (480 mg / ml, Table 3 result) who exhibited no bacteria colonies growing when carried a pourplate method of MIC test at the solid medium [15].The amount of bacteria asserted tend to declined from 300 mg / ml of concentration to 480 mg ml. As more rising concentration extract as much more active compounds contained, it gave antibacterial work effectively. Pelczar and Chan ascertained that higher concentration of a antimicrobes more large its ability to hinder or kill microorganisms [20]. At the concentration above 460 mg / mls (480 mg/ ml and 500 mg/ ml) no bacteria colonies be found. As much 0.00070 x10^6 CFU/ml bacteria colonies established at 460 mg/ml concentration of ketapang extract leaves with TSA medium. `

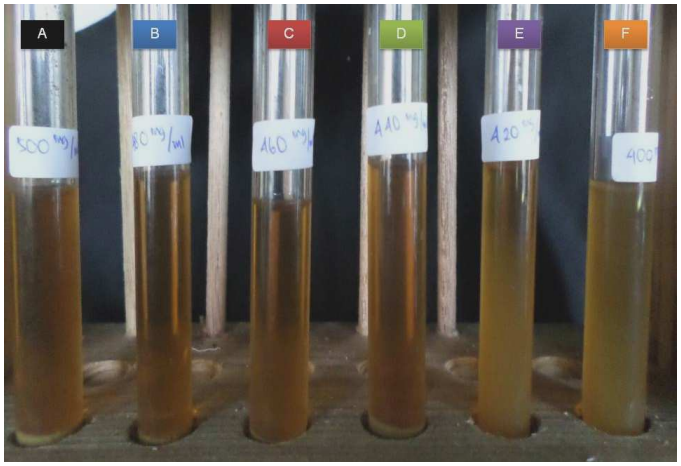

Figure 2. Turbidity of TSB medium with Ketapang leaves extract granting at dilution method (a) 500 mg/ml, (b) 480 mg/ml, (c) 460 mg/ml, (d) 440 mg/ml, (e) 420 mg/ml dan (f) 400 mg/ml.

Bacteria grew so fast after 24 hours of incubation time in TSB media that only added bacteria without extract so that it unable to be counted. While in media that only added extracts without the addition of A. *Salmonicida* bacteria, no bacteria are grown. It affirmed that T. *catappa* leaves extract not contaminated by bacteria or even fungi.

CONCLUSION

The establishment of clear zone on a disk diffusion test method on each concentration informed that T. *catappa* leaves extract proved potentially in against A. *salmonicida*. The higher concentration lead more diameter clear zone had formed .The largest clear zone at 23.167 mm solidified at concentration of 400 mg per mls. Minimum Inhibitory Concentration (MIC) reached at 440 mg per ml and 480 mg per ml for Minimum Bactericidal Concentration (MBC). Separating secondary metabolite compounds are suggested for further research, where these results can be used as guidelines for in vivo research.

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