Jurnal Veteriner
pISSN: 1411-8327; eISSN: 2477-5665
Terakreditasi Nasional, Dirjen Penguatan Riset dan Pengembangan,
Kemenristek Dikti RI S.K. No. 36a/E/KPT/2016

September 2018 Vol. 19 No. 3: 321-328
DOI: 10.19087/jveteriner.2018.19.321
online pada http://ojs.unud.ac.id/index.php/jvet

The Bioactive Compounds of *Avicenia* sp Stem Extract Improved the Viability of Fish Challenged with *Aeromonas hydrophila*

(SENYAWA BIOAKTIF EKSTRAK BATANG AVICENIA SP MENINGKATKAN VIABILITAS IKAN YANG DITANTANG DENGAN AEROMONAS HYDROPHILA)

Alimuddin Ali*[1], Iwan Dini[2], Darminto[2], Hartati[1], Herlina Rante[3]

[1]Laboratory of Microbiology, Department of Biology,
[2]Department of Chemistry, Faculty of Mathematic and Natural Sciences,
Universitas Negeri Makassar.
Kampus UNM Parang Tambung, Jl. Daeng Tata,
Parang Tambung, Makassar, Kota Makassar
South Sulawesi, Indonesia 90224
Email: muddin_69@unm.ac.id Telp: +6281355895039
[3]Laboratory of Microbiology, Faculty of Pharmacy,
Hasanuddin University. South Sulawesi, Indonesia

ABSTRACT

Aeromonas hydrophila is one of bacteria as major diseases for the *Motile Aeromonads Septicemia* (MAS). The aims of this research to get a substance which potential as bioactive of stems bark of *Avicennia* sp. for increase fish viability which exposed of *A. hydropila*. The samples were extracted by using ethanol 70% and then evaluated of antibacterial activities by paper disk diffusion agar technique. The active extracts were partial purified by chromatography column. The fraction of substance was show activities against bacteria analyzed by GC-MS. The results were found four peak and predicted as (1) 2-propenoic acid, 3-[3, 4-dimethoxyphenyl]-methyl ester (2) 3, 4-dimethoxycinnamic acid, (3) 1, 1-dimethyl guanidine (4), Methyl 10-oxostearate. The active fraction was isolated from stem bark of *Avicennia* sp. should be further studied in animal models for *in vitro* efficacy and toxicity. The percentage of *in vivo* towards test fish viabilities showed the difference between treated and untreated. The percentage of viability ranged from 35 to 83.33% while control was 91.67%. Based on finding, we suggest that *Avicenia* sp. bark extract was increasing of fish viability.

Keywords: *Avicennia* sp, *Aeromonas hydrophila*, antibacterial, fish viability

ABSTRAK

Aeromonas hydrophila merupakan salah satu penyakit bakteri utama pada MAS (*Motile Aeromonads Septicemia*). Penelitian ini bertujuan untuk mendapatkan komponen senyawa bioaktif dari ekstrak batang *Avicenia* sp yang berpotensi meningkatkan viabilitas ikan yang diuji tantang dengan bakteri pathogen *A. hydrophila*. Sampel diekstraksi dengan menggunakan etanol 70% lalu dilakukan uji aktivitas antibakteri menggunakan metode cawan kertas. Ekstrak aktif dilakukan purifikasi secara parsial menggunakan kromatografi kolom. Fraksi aktif yang menunjukkan aktivitas antibakteri dilakukan analisis GC-MS. Hasil penelitian menunjukkan bahwa ada 4 puncak yang diprediksi sebagai (1) 2-propenoic acid, 3-[3,4-dimethoxyphenyl]-methyl ester (2) 3,4-dimethoxycinnamic acid, (3) 1,1-dimethyl guanidine (4), Methyl 10-oxostearate. Fraksi aktif yang diperoleh dilakukan pengujian lanjutan efikasi dan toksitas terhadap hewan model secara *in vitro*. Pengujian secara *in vivo* menunjukkan adanya perbedaan secara signifikan antara ikan yang diperlakukan dengan fraksi aktif dengan yang tidak. Persentase viabilitas berkisar 35 sampai 83,33% sedangkan kontrol 91,67%. Temuan ini menunjukkan bahwa ekstrak batang *Avicenia* sp. mampu meningkatkan viabilitas ikan.

Kata-kata kunci: *Avicennia* sp, *Aeromonas hydrophila*, antibakteri, viabilitas ikan

INTRODUCTION

Aeromonas hydrophila and other motile aeromonads are among the most common bacteria in freshwater habitats throughout the world, and these bacteria frequently cause disease among cultured and feral fishes (Cipriano, 2001). The traditional application of antibiotics and chemotherapy has been characterized by partial success in the management of diseases like motile aero monad septicemia (MAS). However, application of antibiotics and chemotherapeutic drugs in the disease management though this practice has triggered the emergence of drug resistant strains in pathogens (Harikrishnan and Balasundaram, 2005). Therefore, application of biologically active substances which isolated from organisms such as plant, animal as natural product is one of good solution for controlling resistance and environment negative effect (Harikrishnan and Balasundaram, 2008).

Mangroves plants is one of organisms which have a biochemically unique, producing a wide array of novel natural products. Different parts of the plant are used in the indigenous system of medicine for the treatment of various human ailments. Researchers have isolated a variety of other mangrove compounds including taraxerol careaborin and taraxeryl cis-p-hydroxycinnamate from leaves of *Rhizophora apiculata* (Kokpol *et al.*, 1990), triterpenoids from leaves of *Rhizophora* species (Dodd *et al.*, 1995); pimaren diterpenoids from, *Bruguiera gymnorrhiza* (Han *et al.*, 2005): iridoid glycosides from leaves of *Avicennia officinalis* (Sharma and Garg, 1996), and novel terpenoids and alkaloids of medicinal *from Excoecaria agallocha* Linn (Satyan *et al.*, 2009) and azadirachtin on non-specific immune parameters of goldfish (*Carassius auratus* Linn.1758) (Kumar *et al.*, 2013). Mangroves are also rich in polyphenols and it's have long been used in folk medicine to treat disease (Bandaranayake, 1998), antifouling compounds (Chen *et al.*, 2008), as modulators of the multidrug resistance (Ma *et al.*, 1998)

In this study, *Avicennia* sp. extract displayed some degree of antimicrobial activity especially against *A. hydrophila*. The present study was undertaken to investigate the in vitro antibacterial activity of ethanol and ethyl acetate extracts from leaves, roots and stem barks of *Avicennia* sp.

RESEARCH METHODS

All other ingredients used were of analytical grades. Fish, nile (9.2±1.7 g) were obtained from Fish Breeding Center, fish feed was obtained from Aquaculture Specialty, solvent and silica gel was obtained from General Labora, Yogyakarta, Indonesia.

Preparations of extracts

The samples were collected from different mangrove ecosystems of South Sulawesi Province. The extract from leaves, roots and stem bark of plant were prepared by maceration adding 3.30 Kg of powder plant to solvent at room temperature and eluted with n-hexane, ethyl acetate, chloroform, aceton, and methanol to get sub-fractions.

The solvent was separated by separating funnel flask and removed under vacuum at 40°C and concentrated till dryness. All extract was assayed for their antimicrobial activity using respective solvent as control by agar diffusion paper disk methods. The fractions showing the highest antimicrobial activity were pooled and subjected to a second silica gel column chromatography.

Fractionation of extracts

Column chromatography and thin layer chromatography performed fractionation steps. The column (50ml) was filled with silica gel (mesh 60-120) and then eluted stepwise with solvent system was increased polarity. The activity was measured for all fractions obtained from the above process with different solvent mixtures. Then the active fractions obtained from the different solvent mixtures were further purified by thin layer chromatography using silica gel (mesh 60).

Antibacterial activity assay

A. hydrophila was using in this study obtained from Faculty of Aquiculture, Gadjah Mada University, Yogyakarta, and grown in Trypticase soybean broth at 37°C and maintained on nutrient agar slants at 4°C. For detection of antimicrobial activity, paper disk was saturated with crude or pure extract was used as antibacterial and placed on Heart Infusion Bouillon agar media (pH 7) after seeded inoculum containing 10^6cfu/mL and then incubated at 37°C for 24 h by diffussion agar tehnique. The activity of substance was

measured in diameter of the clear zone and expressed as mm of inhibition.

Antibacterial susceptibility testing

The minimal inhibitory concentrations (MICs) of all the extracts were determined by micro dilution techniques in Mueller-Hinton broth. Inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard [10^6 colony-forming units (CFU)/ml] and diluted 100-1000 ppm with DMSO for the broth micro dilution procedure. Micro titer plates were incubated at 37ºC and the MICs were recorded after 24 h of incubation. Two susceptibility endpoints were recorded for each isolated. The MIC was defined as the lowest concentration of compounds at which the microorganism tested did not demonstrate visible growth.

Formulation of fish feed

Pellet of fish commercial feed was subsequently crushed with a pestle in a mortar, then mixed with plant extract according to type of treatment. The mixture was supplemented with an adhesive arabic gum 5% and formulated again into pellets.

***In vivo* assay of bioactive substances**

In vivo assay was conducted on compounds which the highest inhibitory activities to determine the viabilities of tested fish (*Oreochromis niloticus*). Healthy Nile tilapias (9.2±1.7 g) were obtained from Fish Breeding Center, Yogyakarta. The study was carried out in thriplicate (three aquariums per experimental groups) with allocated in 5 L of water (seven fish/ aquarium). The tested fish were acclimatized for five days in fresh water. The bioactive substances were applied if the tested fish mortality does not exceed 3%. The experimental was designed according to Angka *et al*., 2002.

The test fish were maintained in the aquarium container equipped with aerator pump. Avicennia bioactive fractionized was added to the feed at 0 g/Kg as control (C0); 0.1% (g/Kg feed)(A); 0.5% (g/Kg feed); 1%(g/Kg feed); 1.5%(g/ Kg feed); 2%(g/Kg feed); and 2.5%(g/Kg feed). The test fish were fed with 2.5% of body weight during the trial. Water was changed daily at rate of 10% of total volume. Fish maintenance was carried out for 20 days and behavior was observed before and after introduction of bacteria. Ten (10 days) before final experimentally, the fish were exposed *A. hydrophila* at 10^6cfu/ml. During the experimental period the main parameter of water were measured as; temperature (25±0.70°C, pH 7.7±0.5 and dissolved oxygen 6±0.8 mm/L).

Statistical analysis

Statistical analysis of the data involved analysis of variance followed by LSD. Differences were considered significant at pÂ0.05

RESULTS AND DISCUSSION

Based on these research were found that different plant were obtained for the studied extracts against test bacteria. The ethanol extracts of mangrove plant especially of *Avicennia* sp. and *Terminalia catappa* leaf, stem bark and roots were active against test bacteria. On the other hand, genera *Rhizophora* spp and *Nipah* spp not shown good activity against tested bacteria (Table 1).

The result of analysis of chemical content by fractionated column chromatography especially ethanol stem bark of *Avicennia* sp. were shown that secondary metabolite of extract founded several component such as terpenoid, steroid, flavonoid and alkaloid.

The active ethanol extract from stem bark of *Avicennia* sp. was submitted to vacuum chromatography over on silica gel to give 23 fractions A (1.9652 g), B (0.9015 g), C (0.8810g), D (0.0830 g), E (1.9550g), F (2.7180 g). The fractions A to F were assayed against *A. hydrophila* by diffusion agar technique (Table 2).

Although the differences were not significant, the stem bark ethanol of *Avicennia* sp. extracts tended to be more active than the leaves and roots extracts after purified (Data not shown). The results showed that bark material could be useful for antibacterial uses, and it could be used although any detrimental effect on the plant. According to Reverter *et al*., 2014 plant derived extracts against bacteria and reported their use in reducing of fish mortality in fish culture or an alternative to chemotherapy.

The following fractions A which exhibited antibacterial properties, was further fractionated by pressure column chromatography on silica gel and then using for primary studied. The result of chromatography on silica gel and the resulting sub-fractions were analyzed by TLC and the combined fraction (Table 3).

Table 1. Antibacterial activity (*A. hydrophila*) of several mangrove plants organ

Plant /Organ	Activities of extract against *A. hydrophila*			
	n-hexane	ethylacetate	ethanol	methanol
Stem bark of *Rhizophora apiculata*	-	-	+	-
Fruits of *Rhizophora apiculata*	-	-	-	-
Roots bark of *Rhizophora apiculata*	-	-	-	-
Leaves of *Rhizophora apiculata*	-	-	-	-
Stem bark of *Rhizophora* sp	-	-	-	-
Seed of *Rhizophora* sp	-	-	-	-
Leaves of *Rhizophora* sp	-	-	-	-
Leaves of *Avicennia* sp	-	+	(+++)	-
Fruits of *Avicennia* sp	+	+	(+++)	-
Stem bark of *Avicennia* sp	-	+	(+++)	-
Roots of *Avicennia* sp	-	-	(+++)	+
Stem bark of *Terminalia* sp	+	+	(++)	-
Stem bark of *Nipah* sp	-	-	(+++)	-
Control of solvent	-	-	-	-

(+++) : high activity (e”15 mm)
(++) : moderate activity (e”10 mm)
(-) : no activity

Table 2. Dry weight of combined fraction of ethanol extract fractionation from *Avicennia* sp. stem bark.

Identities of combined fraction	Combined fraction:	Weight(g)	Properties
A	Fraction 1-4	1.9652	Dilute in ethyl acetate with little of n-hexane or chloform- ethyl acetate
B	Fraction 5-6	0.9015	Dilute in ethyl acetate orchloroform or mixed both
C	Fraction 7-10	0.8810	Dilute in ethyl acetate and acetone
D	Fraction 11-13	0.0830	Dilute in aceton and methanol
E	Fraction 14 – 20	1.9550	Dilute in methanol and acetone
F	Fraction 21 – 23	2.7180	Dilute in methanol

The following purification was done from fraction A5 of substances which activity against bacteria by GC-MS were found eight peak and assumed as (1) 2-propenoic acid, 3-[3,4-dimethoxyphenyl]-methyl ester (2) 3,4-dimethoxycinnamic acid, (3) 1,1-dimethyl guanidine (4), methyl 10-oxostearate. The purification yielded (fraction A5) more active against tested bacteria were purified by using solvent for recrystallization.

The fraction A5 was formed white-green crystal and purified with using nonpolar solvent (n-hexane-ethyl acetate) until to get white crystal. The substances were purified analyzing by three eluent system and shown a homogen single spot. The properties of substances were melting point at 248°C with ranges 248-250°C. The results of antimicrobial activities of the extracts by using both micro dilution assay (MICs) are achieved that $4.5x10^{-3}$ g/mL. However, the extracts and active compound isolated from stem bark of *Avicennia* sp. should be further studied in animal models for in vitro efficacy and toxicity. In term of conservation,

Table 3. Combined of fraction A based on TLC analyzed

Identity of extract	Combined of fraction:	Weight (g)
A1	Fraction 1-10	0.0739
A2	Fraction 11-17	0.5540
A3	Fraction 18-38	0.0366
A4	Fraction 39-59	0.2901
A5	Fraction 60-69	0.4782
A6	Fraction 70-97	0.4775
A7	Fraction 98-112	0.0450

Table 4. The result of GC MS analyses of fraction A5.

Peak	Retention Time (RT)	Area%	Prediction of Substances
1	21.112	15.57	3-[3,4-dimethoxyphenyl]-methyl ester
2	21.327	14.15	3,4-dimethoxycinnamic acid
3	23.194	6.41	1,1-dimethyl guanidine
4	**23.512**	**11.77**	Not determined
5	24.957	16.51	Methyl 10-oxostearate
6	26.970	7.13	Not determined
7	**30.385**	**9.23**	Not determined
8	**30.643**	**19.22**	Not determined

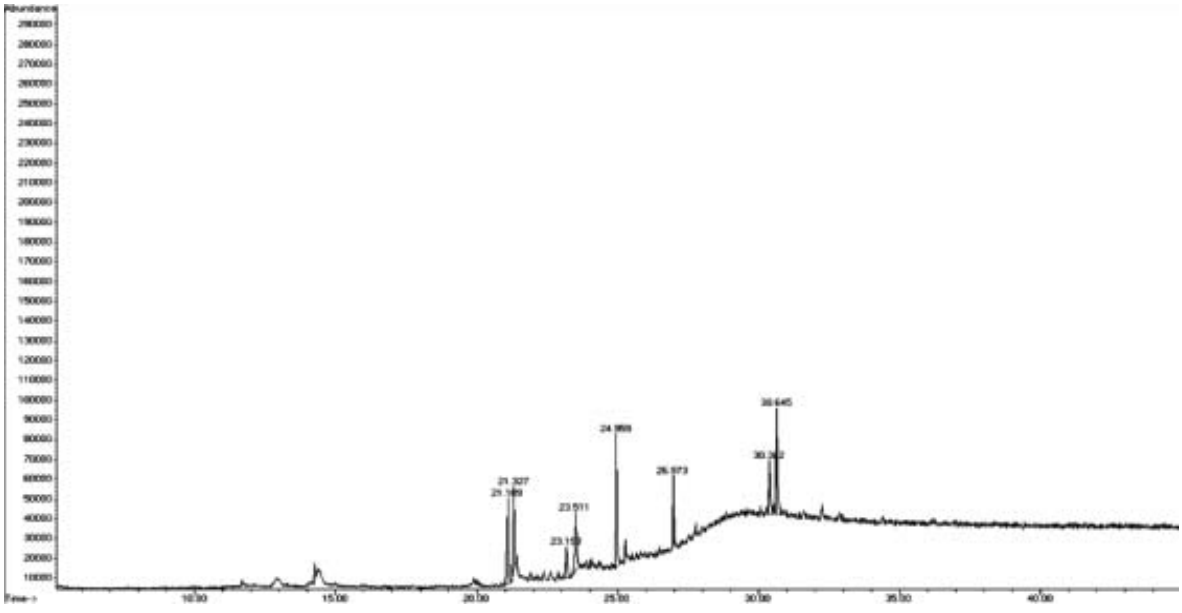

Figure 1. Chromematogram of GS-MS from fraction A5

the results showed that leaf material could be useful for antimicrobial uses, and it could be used without any detrimental effect on the plant.

Based on the IC_{50} values were obtained from each of the components of bioactive components A, B, C and D are 619.44; 371.54; 1.19674 and 221.820 µg/ml, respectively (Data not shown). Of these, the IC_{50} values for all the bioactive components are above 100 µg/ml as a criteria of the inhibition of normal cells. Eventhough that the bioactive component C which a very high IC_{50} value showed less adverse effects of the tested fish.

There are two groups of tested fish treatment i.e treated bioactive compound and inoculated

bacteria), while the other group was untreatment (untreated bioactive compounds and uninoculated by bacteria) were used as controls. The percentage of the tested fish viabilities showed the difference between treated and untreated. The percentage of survival of the tested fish range to 35±2.15 to 83.33±7.92, whereas control 91.67±10.73. In this present study, the bioactive component of D, E, F treatment and control are significantly at the level of 99% (Table 2). It is shows that the bioactive component ability to increase the survival of tested compared with bioactive componen A, B and C treatment. Water quality parameter was no significantly difference in temperature in the control and treatment water. However, there were variations water quality parameters of dissolved oxygen was between treatment group and control, while pH ranged from 6.9 to 7.4 as presented in Table 5.

The ability of inhibition against tested bacteria of each component by the partial purified of substances show that the plant extract potentially as a phytopharmacological candidate. Therefore, the bioactive components of the extract may potential to kill or disturb when applied to fish. To knowing the extent of these components does not cause harmful effects to the fish, IC_{50} of the component was examined against normal cells (Vero). The results showed that all the bioactive components, none of the compound potentially harmful to normal cells.

A. Avicena bioactive fractionized was added to the feed at 0.1% [g/Kg feed] and exposed bacteria; B. 0.5% [g/Kg feed] and exposed bacteria; C. 1% [g/Kg feed] and exposed bacteria; D. 1.5% [g/Kg feed] and exposed bacteria; E. 2% [g/Kg feed] and exposed bacteria; F. 2.5 % [g/Kg feed] and exposed bacteria; K. 0 % [g/Kg] and unexposed bacteria. The values in the same

Table 5. Viability of tested fish after treated by bioactive compounds against *A. hydrophila* after eight days exposed plant extract

Treatment group	Viability of tested fish (%)	Final pH	Temperature (°C) of water	Dissolved Oxygen (ppm)
A	$35^{a} \pm 2.15$	7.3	29.5	5.7
B	$41.67^{a} \pm 3.66$	7.1	28.8	5.9
C	$48.33^{a} \pm 5.64$	7.4	29.2	4.8
D	$51.67^{a} \pm 6.13$	6.9	29.1	5.9
E	$75^{b} \pm 3.51$	7.2	28.9	4.3
F	$83.33^{cb} \pm 7.92$	6.9	28.6	5.5
K	$91.67^{cd} \pm 10.73$	7.2	29.3	7.1

A B

Figure 2. The profile of condition of fish untreatment with plant extract, exposed bacteria (A) and treatment with plant extract, exposed bacteria (B)

column followed by the same lowercase letter are not significantly by the Least Significant Difference $a_{0.05}$

The low rate of mortality observed for exposure bacteria of fish treated by extract shows that the herb may be effective in the control of pathogen bacteria in fish culture. The concentration of extract was observed influence against mortality of fish. High dose application of the herb can be of immediate benefit for effective control of bacteria and increasing of viabilities in fish culture

The visual observation of the tested fish which treated of bioactive component D, E, F and control shows the condition of the fish is much healthier compared with three other treatments. The physical condition of the water more clear and does not cause unpleasant odors. Meanwhile, if compared with A, B and C, physical condition of water appears very cloudy and smelled dreadful. This is caused by the growth of bacteria population were uninhibited by the bioactive component.

According to Falkinham *et al.* (2015) opportunistic pathogen of *A. hydrophila* ability causing disease influenced by high populations of fish. This means that the number of fish population has linier correlation with prevalence of the disease.

Furthermore, clinical symptoms of fish show different beetwen treated with plant extract compared untreated plant extract but exposed bacteria (Figure 2A). The skin is very pale and quite a lot of mucus on the skin surface compared untreatment plant extract, skin are bright and does not indicate the presence of excess mucus (Figure 2B). It was might to indicate that the fish was stressing larger than others.

Many kinds of herbs had been introduced to fish suffering from infection diseases. For example plant extract has been mixed to the common crap (*Cyprinus carpio*), pellet and feed every day to protect the bacterial infection (Harikrishnan *et al.,* 2003; Mohomad and Abasali, 2010). Effect of *Rehmannia glutinosa* on growth performance, immunological parameters and diseases resistances to *A. hydrophila* in common carp was reported by Wang *et al*. (2015). Harikrishnan and Balasundaram (2008) found that the herbs extract, a naturally occuring antimicrobes from subtances plant showed significantly in vitro antibacterial activitity against isolates of fish pathogen *A. hydrophila.*

CONCLUSION

The present study showed that *Avicennia* sp. bark extract has a potential value for aquaculture both in terms of increasing viabilities of fish in culture or anti-pathogenic bacteria.

SUGGESTION

We suggest to studying of *Avicenia* sp. bark extract beneficial on physiological response like immunity, and hematology of fish was exposed this herbs extract.

ACKNOWLEDGEMENTS

These research was supported by grants from the Hibah Bersaing Program, Dikti 2010. We are grateful to Drs. Nanang Besmanto, M.Si (Balai Teknik Kesehatan Lingkungan, Yogyakarta) and our student for help in this research.

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