Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

p-ISSN: 0972-6268 e-ISSN: 2395-3454	Vol. 16	No. 1	pp. 231-235	2017

Original Research Paper | Open Access

Effect of Crude Ethyl Acetate Extract and Fraction of Soursop (*Annona muricata*) Leaf on Morphology of *Edwardsiella tarda* with Scanning Electron Microscopy

Happy Nursyam*† , Madyasta Anggana Rarassari* and Maftuch**
*Laboratory of Microbiology, Faculty of Fisheries and Marine Science, Universitas Brawijaya, Indonesia
**Laboratory of Fish Disease, Faculty of Fisheries and Marine Science, Universitas Brawijaya, Indonesia
†Corresponding author: Happy Nursyam

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 22-02-2016
Accepted: 16-04-2016

Key Words:
Antibacterial
Acetate fraction
Annona muricata
Edwardsiella tarda
Anthraquinone

ABSTRACT

The objective of this research was to assess the antibacterial activity of ethyl acetate fraction extract of *Annona muricata against Edwardsiella tarda*, knowing antibacterial compounds and structural changes of *Edwardsiella tarda* by using Scanning Electron Microscopy. Method of this research used was descriptive for estimating the results of the compound in the ethyl acetate fraction of *Annona muricata* and morphological changes of *Edwardsiella tarda*, as well as experimental antibacterial activity seen from the zone of inhibition. Data obtained from the results of further studies were statistically analysed used by statistical applications. Result showed that the yellow fraction of the crude extract ethyl acetate produced the best inhibition was 9:35 mm, and MIC with a concentration of 62.5 ppm. Estimation compound ethyl acetate fraction of *Annona muricata* by LCMS results that corresponded to the type of compound classes anthraquinone emodin. Ethyl acetate fraction of *Annona muricata* showed a relatively moderate inhibitory activity against bacterial pathogens, it was *Edwardsiella tarda*, as well as an optimal compound act as an antibacterial can already be identified, it was from phenol derivatives.

INTRODUCTION

Edwardsiella tarda is bacteria on the fish caused of Edwardsiellosis. These bacteria move with the flagella, do not form spores or encapsulated and live in freshwater environments (Austin 1999). *Edwardsiella tarda* is Gram-negative, short, rod-shaped, facultative anaerobes, 2-3 µm long and with a diameter of 1µm (Park & Jung 2012). Edwardsiliosis is a bacterial disease that is a very serious systematic cultivation of eels (Japanese eel) in Japan and Taiwan, Japanase flounder fish and other farmed fish. In the USA, *Edwardsiella tarda* caused of septicemia infectious diseases in fish channel catfish (*Ictalurus punctatus*), host and geographic distribution (Ikhsan 2013). Therefore, necessary reduction or control of edwardsiellosis caused by bacteria *Edwardsiella tarda* is required.

Bacterial infectious disease problems can be resolved with the fish health management through control efforts. Control is usually done is by the administration of drugs or antibacterial substances such as antibiotics through the activities of prevention (preventive) and treatment (curative) (Kordi 2004). Efforts should be made in the control of bacterial infections of *Edwardsiella tarda* using antibacterial synthetic or natural, but the use of synthetic antibacterial (antibiotic) actually counter productive because the impact caused the antibiotic residues in tissues (Wattimena et al. 1991) which causes a decrease in the quality of the product, i.e. impairment food security. Antibiotic residues can be passed to humans who consume them, so that the necessary effort to get antibacterial of natural ingredients that can be used to control bacterial pathogens (Riniatsih et al. 2007).

Antimicrobial activity was also produced by plants is known as fitofarmaka; use fitofarmaka in Indonesia has long been used because abundance of antimicrobial potency from natural ingredients that are safer but have functions and activities that are not less of antibiotics (Darminto et al. 2009). One antibacterial of natural plant materials and can be used bioactive compounds from the leaves of the sour sop (*Annona muricata*). Octavia & Leni (2003) indicated that sour sop leaves are known to contain active compounds that are toxic, this situation allows soursop can be used as an antibacterial compound. From phytochemical analysis of soursop leaves their secondary metabolites are such as tannins, steroids, glycosides, alkaloids, saponins and flavonoids (Wisdom et al. 2014). It can be said that there are antimicrobial compounds in this plant species. From the research results it will be expected to know the antibacterial activity of ethyl acetate fraction extract of *Annona muricata*

against *Edwardsiella tarda* and know antibacterial compounds and morphological changes of *Edwardsiella tarda* by using Scanning Electron Microscopy.

MATERIALS AND METHODS

Preparation extraction of *Annona muricata* leaves: *Annona muricata* leaves were obtained from Malang, East Java, Indonesia. Making of *Annona muricata* leaf extracts used by maceration method. Extraction of *Annona muricata* leaves was used by maceration method with solvent ethyl acetate (1: 3 w/v) allowed to stand for 48 hours at room temperature. The filtrate was separated from the waste with filtered used by Whatman paper No. 42. The filtrate, then evaporated with a vacuum evaporator at a temperature of 60-70°C until all the solvent evaporates or did not take place again in the condenser. The next step is weighed heavy extracts.

Chromatography columns: The working principle of the separation column chromatography column filled with absorbed solids such as alumina (fixed phase) and fed with solvents such as benzene (mobile phase) (Sastrohamidjojo 2007). Separation of antibacterial compounds ethyl acetate extracts of *Annona muricata* leaves performed was using column chromatography, fractionation column chromatography antibacterial compounds was using 70-230 mesh silica gel as stationary phase while the mobile phase used by ethanol (polar) and chloroform (non-polar) with comparison that has been modified 100: 0, 75:25, 50:50, 25:75 and 0: 100. The stationary phase was made by mixed silica gel with ethanol at a ratio of 1: 2 (w/v). Then silica gel and ethanol stirred, the supernatant discarded. Ethanol added with a ratio of 1: 1, stirred back up into the form of slurry. The slurry was slowly inserted into the column and left for 15 minutes. After the liquid extract as much as 3 mL put in a piecemeal column eluted with a comparison between ethanol and chloroform which the mobile phase.

Antibacterial activity trial: The method used to determine the fraction of ethyl acetate extract was most effective as an antibacterial *Edwardsiella tarda* using a paper disc diffusion method. Paper disc diffusion method, known as Kirby-Bauer method used to determine known antibacterial sensitivity by measuring the inhibition zone or a clear zone formed around the paper discs (diameter 6 mm) which already contained antimicrobial substances.

Test of inhibitory fraction from ethyl acetate extract against bacteria *Edwardsiella tarda* of the process that began with both bacterial culture prior to medium prepared TSA (Triptic Soy Agar) and medium Triptic Soy Broth (TSB) for bacteria *Edwardsiella tarda* then performed bacterial cultures by inoculating bacteria on solid medium with distributed evenly using a sterile cotton swab aseptically. After that, paper disc that has been immersed in each extract for 15 minutes was taken and slowly placed or affixed on top of the solid medium. Then incubated at a temperature of 35°C for 24 hours. Determination of inhibitory fraction of ethyl acetate extract of *Annona muricata* leaves done by measuring the diameter (mm) clear area around the paper discs using a caliper.

Determination of value MIC (Minimum Inhibitory Concentration): Fractions of ethyl acetate *Annona muricata* leaves extract produced the best inhibition against *Edwardsiella tarda* then performed for determining the MIC (Minimum Inhibitory Concentration) in various concentrations of 1000 mg/L, 500 mg/L, 250 mg/L, 125 mg/L, 62.5 mg/L, 31.2 mg/L, 15.6 mg/L and 7.8 mg/L. The best fractions of ethyl acetate *Annona muricata* leaves extracts with various concentrations of the test inhibitory power against *Edwardsiella tarda* and measured the diameter of clear zone formed after incubation for 24 hours at a temperature of 35°C.

Statistical analysis data: The research data were statistically analysed used by ANOVA to see the antibacterial activity of ethyl acetate fraction of *Annona muricata* extract against *Edwardsiella tarda*.

RESULTS AND DISCUSSION

Antibacterial test of ethyl acetate fraction: The fractions resulted of ethyl acetate *Annona muricata* leaves extract amounted to 5 fractions were tested for antibacterial activity against bacteria *Edwardsiella tarda* using the disc. Re-

Tabel 1: Fractions resulted of ethyl acetate *Annona muricata* leaves extract and inhibition against bacteria *Edwardsiella tarda.*

Fraction Codes	Eluen comparison (Ethanol:Chlorofom)	Colour	Inhibition zone (mm) ($\bar{x}$ ± sd)
F1	100:0	Clear	0
F2	75:25	Thick green	7.76 ± 0.289, 35 ± 0.19
F3	75:25	Yellow	
F4	50:50	Clear	0
F5	25:75	Clear	0
F6	0:100	Clear	0

sults fractionation of ethyl acetate *Annona muricata* leaves extract and inhibition against bacteria *Edwardsiella tarda* can be seen in Table 1.

Tested of antibacterial activity from all fractions of ethyl acetate *Annona muricata* leaves extract showed that the yellow F3 fraction has the greatest inhibition zone diameter of 9.35 mm, followed by fraction F2 thick green with inhibition zone diameter of 7.76 mm. But the continued fraction for further analysis is the fraction F3 in yellow, because the fraction has inhibitory larger when compared with other factions. Qualitatively group of plants yellow pigment belongs to the class of cytochrome compounds, carotenoids, anthraquinone, flavonols, khalkon and Auron (10), but needed further tests to determine the compound of yellow *Annona muricata* leaves fraction. Phenol and derivatives bind to the protein through hydrogen bonding resulted protein structure became damaged. Where most of the structure of the cell wall and bacterial cytoplasmic membrane protein and fat. Instability in the cell wall and cytoplasmic membrane of bacteria causing selective permeability function, active transport function, controlling the composition of proteins from bacterial cells to be disrupted, which would result in the escape of macromolecules, and ions from the cell. So the bacterial cells became lost its shape, and their lysis (Susanti 2008).

Minimum inhibitory concentration test (MIC): F3 fraction then performed by determination of MIC value (Minimum Inhibitory Concentration) against growth of *Edwardsiella tarda* which can be seen in Table 2.

MIC values for fraction F3 ethyl acetate of *Annona muricata* leaves extract was equal to 62.5 ppm. This indicated that the value of the smallest concentrations F3 to inhibit bacterial growth *Edwardsiella tarda* is 62.5 ppm. In the MIC test colour change became turbid means indicated that the bacteria are not inhibited, otherwise if discoloration became clear this indicated that the bacteria inhibited. MIC is a way to determine the lowest concentration of materials that are used as a drug that can kill the growth of *Edwardsiella tarda* visually (Harborne 1996).

Tabel 2: Result of MIC test on *Edwardsiella tarda.*

No.	Concentration (ppm)	Growth of *E. tarda*(+/-)
1	1000	+
2	500	+
3	250	+
4	125	+
5	62,5	+
6	31,2	-
7	15,6	-
8	7,8	-
9	TSB + *E. tarda*	-
10	TSB + Ampicilin + *E. tarda*	+

LC-MS analysis: Ethyl acetate fraction of *Annona muricata* extract F3 qualitatively analysed using Liquid Chromatography - Mass Spectrometry (LC-MS) with ion source such as atmospheric pressure chemical ionization (APCI). F3 LC-MS analysis of compounds containing anthraquinone is emodin derivatives. LC-MS results can be seen in Fig. 1.

The LCMS analysis results, for emodin compounds obtained values of m/z (269/224.5) with a retention time (RT) 0:51 and wide area (AA) 50907. Difference between the m/z anthraquinone derivatives on the results of LC-MS analysis of the best fraction of *Annona muricata* extract with standard methods of analysis for emodin anthraquinone compounds with m/z values (268.9/239.1) (Ardiansyah 2007), this is because type of column that used in the best fraction LCMS analysis using extracts of *Annona muricata* hypersil gold (50mm × 2.5 mm × 1,9 µm). In general, use of LC-MS for the analysis of anthraquinone compounds is advantageous over the use of GC-MS. Because it was giving a high efficiency separation and accurate identification (Wu et al. 2013). Anthraquinone is a compound that has potential as an antibacterial in the leaves of *Annona muricata*. Anthraquinone has a very broad range of antimicrobials as well as a source of free radicals, also can cause the protein to lose its function by changing the morphology of the cells and damage the outer structure of bacteria (Fernand 2008).

Scanning Electron Microscopy (SEM): *Edwardsiella tarda* observations indicated that change in the form of bacterial cells before and after treatment was giving the best of crude extract and ethyl acetate fraction from the leaves of *Annona muricata* as an antibacterial material. Observations on compound antibacterial through SEM can be seen in Fig. 2.

Normal cells of *Edwardsiella tarda* (A) were still visible rods, colonize and cell wall is clearly visible. After being treated with extract from the leaves of *Annona muricata* (B) shown with red arrows found in the cell wall formation of protrusions and cell size gets smaller when compared to the size of normal bacterial cells. The formation of small bumps on the surface of bacterial cells for intracellular peptidoglycan withstand high pressure. This bulge was a sign of disruption of the cell wall biosynthesis process due to antimicrobial activity at low concentrations (Putra 2004).

Class of Gram-negative bacteria have a way to protect cell membranes from penetration of the antibacterial ingredients, as Gram-negative bacteria have a relatively thin wall peptidoglycan, and the periplasmic space between the cell wall and membrane. The structure of the outer membrane contains lipopolysaccharide or endotoxin that play a role in preventing the penetration of hydrophobic compounds,

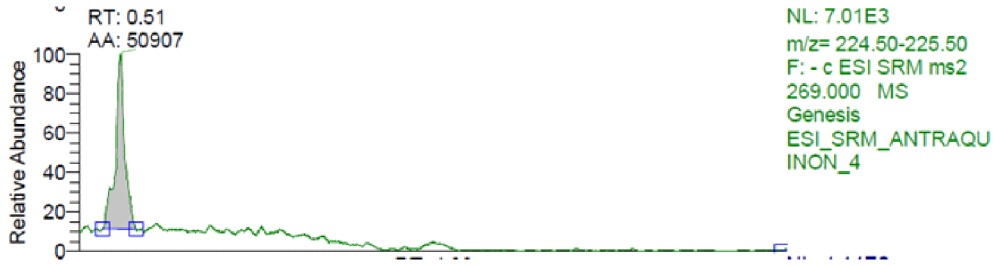
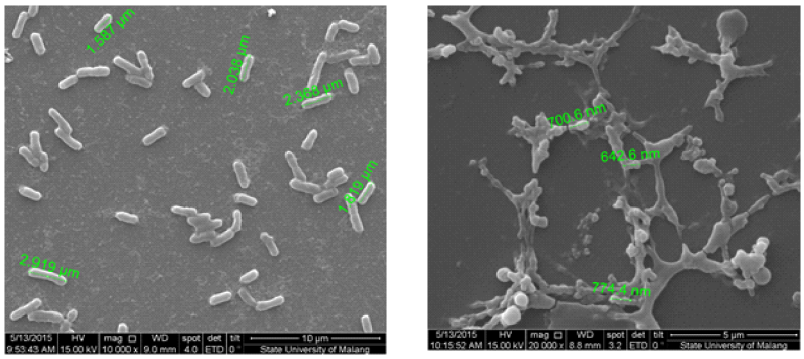


Fig. 1: Chromatogram *emodin* from F3 ethyl acetate fraction of *Annona muricata* extract, y as *relative abundance (%)* danx axis as retention time (minutes).



(A) (B)

Fig. 2: Cell morphology of *Edwardsiella tarda* seen by SEM, (A) *Edwardsiella tarda* cell,(B) *Edwardsiella tarda* cells by treatment with ethyl acetate fraction extract of *Annona muricata* .

such as anthraquinone compounds. In the outer membrane of cells are pores which allow penetration of small molecule-sized compound and hydrophilic just as sugars, amino acids and certain ions (Gilbert 2004). Phenolic compounds from plants have the ability to form a complex compound with the protein through hydrogen bonding, which can damage cell membranes of bacteria (Zhang et al. 2011). However, the presence of a complex cell membrane structure limits active antibacterial compounds such as anthraquinone enter the cell because it allegedly did not have enough levels to penetrate the cell membrane.

CONCLUSION

In this study fraction of ethyl acetate from *Annona muricata* extracts namely F3 showed a relatively moderate inhibitory activity against pathogens *Edwardsiella tarda* amounted to 9.35 mm, as well as allegations identified compounds which act as antibacterial optimal from the class of anthraquinone derivatives of phenol.

REFERENCES

Ardiansyah, A. 2007. Kajian Aktivitas Antimikroba Ekstrak Daun Beluntas, *Plucea indica* L.). (Assessment of Antimicrobial Activity Beluntas Leaf Extract, Pluceaindica L). Thesis. Institut Pertanian Bogor.

Austin, B. and Austin, D. 1999. Characteristic of the diseases. In: Austin B, Austin D (eds) Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish, 3nd edition. London: Springer-Verlag Heidelberg. 13-15.

Darminto, Ali, Alimuddin and Dini, Iwan 2009. Kajian pengembangan dan produksi kandidat fitofarmaka antibakterial pada tumbuhan mangrove untuk pengendalian pengakit bakterial ikan. (Study of the development and production of anti-bacterial fitofarmaka candidates on mangroves for controlling bacterial fish disease). Laporan Penelitian. Pusat Dokumentasi dan Informasi Ilmiah-LIPI.

Fernand, E. V., Dinh, D., Washington, S.J., Fakayode, S., Losso, J., Ravenswaay, R.V. and Warner, I. 2008. Determination of pharmacologically active compounds in root extracts of *Cassia alata* L. by use of high performance liquid chromatography. Journal Talanta Elsivier 74: 896-902.

Gilbert, P. 1984. The revival of microorganisms sublethally injured by chemical inhibitors. In: Andrew MHE, Russell AD, editor. The revival of injured microbes. Academic Press, London.

Harborne, J.B. 1996. Fitokimia. Penuntun Cara Modern Menganalisis Tumbuhan (Phytochemistry. Analyzing the Modern Way guiding Plant). Translated by: Padmawinata, K. Penerbit ITB. Bandung. 354 hlm.

Ikhsan, M. 2013. Upaya Pengendalian Infeksi Bakteri Edwardsiella tarda Pada Ikan Lele (*Clarias* sp) Menggunakan Ekstrak Kunyit, *Curcuma longa*. (Bacterial Infection Control Efforts of *Edwardsiella tarda* in Catfish *(Clarias* sp*)* using the extract of Turmeric, *Curcuma longa).* Thesis. Institut Pertanian Bogor.

Kordi, M. 2004. Pengendalian Hama dan Penyakit Ikan (Pest and Fish Disease Control). Jakarta (ID): Rineka Cipta dan Bina diaksara.

Octavia, Leny. 2003. Uji Antibakteri, Penentuan Kadar Vitamin C, Dan Gula Total Pada Buah Sirsak, *Annona muricata*). (Antibacterial test, Determination Levels of Vitamin C, And Sugar Total At Soursop Fruit, Annona muricata). Journal Kimia Institut Pertanian Bogor. Vol. 2, No.2.

Park, S. B., T. Aoki and T. S. Jung. 2012. Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. Review Journal Veterinary Research: 43-67.

Putra, I N. 2004. Aktivitas Antibakteri Ekstrak Kulit Buah Manggis (Garcinia mangostana L.) Serta Kandungan Senyawa Aktifnya. Jurnal Teknologi dan Industri Pangan. (Antibacterial Activity Mangosteen Extract Skin *(Garcinia mangostana L.)* As well as the content of the active compound). Vol.xx1 No.1.

Riniatsih, Ita dan Widianingsih, 2007. Kelimpahan dan Pola Sebaran Kerang-kerangan (Bivalvia) di Ekosistem Padang Lamun, Perairan Jepara (Abundance and Distribution Patterns of Shellfish (bivalves) at Seagrass Ecosystems, Jepara Sea). Ilmu Kelautan 12(1): 52-58.

Sastrohamidjojo, 2007. Spektroskopi. Liberty. Yogyakarta

Susanti, A. 2008. Daya antibakteri ekstrak etanol daun beluntas (Pluchea indica less) terhadap *Escherichia coli* secara in vitro. (Power antibacterial ethanol extract of "beluntas" leaves (*Pluchea indica* less) against *Escherichia coli* by in vitro method). Jurnal universitas airlangga Vol. 1 No. 1.

Wattimena, J.R., Nelly, C., Sugiarso, Widianto, M.A., Sukandar, E.Y., Soemardji, A.A. dan Setiadi, A.R. 1991. Farmakodinamika dan Terapi Antibiotik (Pharmacodynamics and Antibiotic Therapy). UGM Press. Yogyakarta. 168 hlm.

Wisdom, S., Ugoh, G.O, and Mohammed, B. 2014. *Phytochemical* Screening and Antimicrobial Activities of *Annona muricata* (L) leaf extract. American Journal of Biological, Chemical and Pharmaceutical Sciences, Vol. 2; 44-47.

Wu, Wenjin., Yun, R., Yao, M., Zhan, Y. and Wang, Y. 2013. Pharmacokinetics of anthraquinones in rat plasma after oral administration of a Rhubarb extract. Journal Biomedical Chromatograhy, 28: 564-572.

Zhang, D.Z., Khan, M.A., Chen, H.C and Tania, M. 2011, Anticancer activities of *Nigella Sativa* (Black Cumin), J. Tradit Complement Altern. Med., 8 (S): 226-232.