Antibacterial Activity of Ethyl Acetate the Extract of Noni Fruit
(Morinda citrifolia L.) Against Bacterial Spoilage in Fish

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Abstract. Noni fruit (Morinda citrifolia L.) contains compounds that have potential as antibacterial agent. Antibacterial compounds produced noni fruit (M. citrifolia L.) can inhibit bacterial growth. This study was conducted to test the antibacterial activity of ethyl acetate extract of noni fruit (M. citrifolia L.) against spoilage bacterial in fish. Pseudomonas aeruginosa, Bacillus cereus, Escherichia coli, Klebsiella oxytoca, and Enterobacter aerogenes isolates and examine antibacterial phytochemical profile. Extraction of noni compounds was done by maceration, followed by partition with ethyl acetate to obtain the soluble and insoluble ethyl acetate fraction. Previews result show that the ethyl acetate extract had very strong activity. Extraction process continued by separation and isolation used preparative thin layer chromatography method, so that obtained five isolates and mark them as A, B, C, D and E. Antibacterial activity assay performed on isolates A, B, C, D, and E with 20 and 30% concentration. The test results showed that isolates A could not be inhibit the growth of bacteria, isolates B, C, D, and E has antibacterial activity with weak to strong inhibit. Isolate B had the greatest inhibit ion activity against the B. cereus, whereas isolates E had the greatest inhibition activity against P. aeroginosa. MIC (Minimum Inhibitor Concentration) and MBC (Minimum Bactericidal Concentration) test result showed that MIC and MBC values could not be determined. Analysis of compounds by TLC showed that isolate B suspected contains coumarin or flavonoids compounds that have antibacterial activity.

1. Introduction
Indonesia is a tropical country with high humidity. It can be live to most bacteria, fungi and other microorganisms. Foodstuffs are intermediate or substrate for the development of microorganisms. One of wet semi food ingredients susceptible to decay (perishable food) due to bacterial contamination is fish [1, 2]. The water content was contained in the fish as the main factor causing the damage. Reduction of water content can be done with the compounds which can bind water preservative, as well as limit and kill microbial activity destroyer foodstuffs [3].
Foodstuffs preservation with synthetic preservatives such as benzoic acid, potassium nitrate, BHA (Butylated hydroxyanisole), BHT (Butylated hydroxytoluene) and TBHQ (Tertiary Butylated hydroxyanisole) will cause toxic if prolonged usage [4]. The solution needs to be sought natural preservative that could potentially antibacterial which the opportunity as an alternative to synthetic preservatives.

Noni fruit (Morinda citrifolia L.) is a plant that has antibacterial compounds, such as alkaloids, flavonoids, anthraquinone, scopoletin, glycosides, and glucuronic acid [5]. The study reported that ethanolic extract of noni fruit (M. citrifolia L.) has antibacterial activity with optimum inhibition at 70% concentration [6]. Therefore, it is necessary to separate the isolation of compounds from the dissolved ethyl acetate which have higher activity than the insoluble one. This study focus in the antibacterial activity of noni fruit isolate (M. citrifolia L.) on P. aeruginosa, B. cereus, E. coli, K. oxytoca and E. aerogenes. The final test was conducted to determine the value of MIC and MBC of isolates with the most active concentration.

2. Experimental

2.1 Isolation of Section Ethyl Acetate with Preparative Thin Layer Chromatography (TLC)
Ethyl acetate fraction was isolated using TLC. GF254 silica gel plate was made by 7 grams of powdered silica and 14 mL of distilled water (1: 2), TLC preparation in a glass plate activated by oven on 50°C ± 1 hour. Samples then spotted on a TLC 3 times. TLC elution were done by using (chloroform: ethyl acetate 3: 1) as mobile phase. The formed spot scraped to obtain isolates. Silica gel and isolate then separated using methanol: chloroform with the ratio 1: 1 then filtered to obtain a filtrate. The filtrate is dried into powder and weighed.

2.2 Thin Layer Chromatography and Determination Compound of Isolation Results
Isolate analysis was determined by TLC. Silica gel GF254 used as the stationary phase while chloroform: ethyl acetate (3: 1) used as the mobile phase. TLC analysis then identified under UV (254 nm and 366 nm). Furthermore, the TLC plate was sprayed with cerium (IV) sulphate to detect the presence of general compounds with FeCl₃ and AlCl₃ and then calculated the Rf (retention factor).

2.3 Test of Antibacterial of Isolates Noni Fruit (M. citrifolia L.)
2.3.1 Well Diffusion Method
Sterile media of MHA 10 mL and inoculum poured in the petri dish where inoculated with the bacteria of P. aeruginosa, B. cereus, E. coli, K. oxytoca, and E. aerogenes. Then made sinks with perforator. 25 microliters sample with a concentration of 20 and 30% put on pitting, each concentration of 2 replicates. Furthermore, incubated at 37 °C for 24 hours.

2.3.2 Test of MIC and MBC
Isolates with optimum concentration 40 mg/ mL (2x of the optimum concentration). 50 mL of isolates and 50 mL inoculum were mixed in eppendorf. Then, incubated for 24 hours at 37°C. After 24 hours, the result of incubation displacement in a microplate which contains media of MHA, 25 mL for each repetition, then leveled with glass spreader. Then, incubated for 24 hours at 37°C. If showed bacterial growth were expressed as the Minimum Inhibitory Concentration (MIC). While the absence of bacterial growth was expressed as the Minimum Bactericidal Concentration (MBC) [7].
3. Result and Discussion

3.1 Separation and Isolation Compound

1.7 g isolate from ethyl acetate fraction separated and isolated using TLC method. The result isolation of ethyl acetate soluble 70% on UV 254 showed that 5 isolates could be separated as isolates A, B, C, D, and E. Isolate B has the greatest weight which indicated contains the major compound in noni fruit (M. citrifolia L.).

![Figure 1](image)

**Figure 1.** Profile chromatogram of the isolates A, B, C, D and E with (a) UV254 (b) UV366

The result of isolates then identified 5 bacteria. The bacteria is *B. cereus* as gram-positive bacteria and *P. aeruginosa*, *E. coli*, *K. oxytoca*, and *E. aerogenes* as gram-negative bacteria.

3.2 Test of Antibacterial

3.2.1 Well Diffusion Method

Isolates with the effective concentration of partition 20 and 30% were identified in five bacteria. The bacteria are *P. aeruginosa*, *B. cereus*, *E. coli*, *K. oxytoca*, and *E. aerogenes*. Test of the plate was incubated at 37°C for 24 hours used amoxicillin controls 0.25%, CMC 1% and 70% ethyl acetate.

![Figure 2](image)

**Figure 2.** The antibacterial activity test result with the largest inhibition zone.

The inhibitory activity shown isolates B at a concentration of 30% against the bacteria *B. cereus* by 10 mm were considered to have moderate activity and isolates E at a concentration of 20% against the bacteria *P. aeruginosa* equal to 12 mm is considered to have strong activity. In general, B isolates thought to have compounds that can inhibit gram-positive bacteria and especially *B. cereus*. *B. cereus* is a gram-positive bacterium that is composed of peptidoglycan cell wall more (90%) than the lipid [3, 8]. The cell wall of
gram-positive bacterial contain polysaccharides which are water-soluble polymers, serves as a positive ion transport. The nature of this water soluble indicating that the cell wall of gram-positive bacteria is polar.

Table 1. The results of measurement of inhibitory zone of isolates noni fruit A, B, C, D, E

| Isolates | E. coli 20% | B. cereus 20% | K. oxytoca 20% | E. aerogenes 20% | P. aeroginosa 20% |
|----------|-------------|---------------|----------------|-----------------|------------------|
| A        | -           | -             | -              | -               | -                |
| B        | 3           | 4             | 10*            | 4               | 4                |
| C        | 4           | 4             | 5              | 4               | 6                |
| D        | 1           | 2             | 2              | 3               | 5                |
| E        | 4           | 5             | 6              | 5               | 12*              |

Amoxicillin 2.5%: 36 29 24 16 28
CMC 1%: 0 0 0 0 0
ethyl acetate 70%: 0 0 0 0 0

Description: * The size of inhibition zone of each isolate

P. aeroginosa bacteria is gram-negative bacteria which is composed of layers of peptidoglycan in the cell wall is thinner than the lipid [3, 8]. The peptidoglycan layer builds structures and defenses of bacteria, which can affect the mechanism of inhibition of antibacterial compounds. The mechanism of inhibition was common in the antibacterial compound that inhibits cell wall synthesis. All cells, including bacteria, have a cell membrane that allows the movement of substances in and out of the cell with a controlled way. Antibacterial compounds can affect the integrity of cell membranes, cause damage or even death of the bacterial cell [9]. Inhibitory activity was shown the results of isolates ranges from weak to strong, but overall isolates antibacterial B, C, D, and E have a broad spectrum of activity. It can inhibit the growth of bacteria positive and negative.

3.2.2 Test of MIC and MBC

The test was performed on the isolates had bacterial growth inhibitory activity on antibacterial agar diffusion test. From the results of the concentration of 20% and 30% showed no significant. Then, the test of MIC / MBC used a concentration of 20%. The value of MIC was determined by the absence of bacterial growth media test after incubation of 24 hours at a temperature of 37 °C which it showed the presence of growth after being transferred to a new medium. And the value of MBC was determined by the absence of growth after incubation of 24 hours at a temperature of 37 °C or after being transferred to new media with certain concentration.

The test results of MIC/ MBC show all the bacteria grow at a concentration of 20% after 24 h incubation at a temperature of 37 °C. This means isolates B, C, D, and E with a concentration of 20% can not be bacteriostatic or MBC. According to Liana [10], fraction 6 (F6) of methanol extract of the “Senggani” leaves are bacteriostatic at concentrations above 300 mg / mL against Staphylococcus aureus bacteria. It means that the characteristic of bacteriostatic or bactericidal affected by concentrations of antibacterial and conditions the bacteria. Antibacterial bacteriostatic works to inhibit the growth of bacteria, while bactericidal works to kill bacteria [11].
3.3 Detection compound of Isolates with Reagent Spray and UV

Detection of compounds with spray reagent FeCl$_3$, AlCl$_3$ and UV rays performed on all isolates to suspect compounds in each isolate. Results of detection isolate A, B, C, D, E shown in table 2. And the result chromatogram of spray reagent shown in figure 3.

Table 2. Results of detection of isolates A, B, C, D, E with reagent spray

| No | Isolate | Reagent Spray | Hypothesis                  |
|----|---------|---------------|------------------------------|
| 1  | A       | Light yellow  | Reddish yellow               | Phenol                        |
| 2  | B       | Black brown   | Yellow                       | Flavonoid or coumarin         |
| 3  | C       | -             | blue phosphorescent          |                              |
| 4  | D       | -             | Blue                         | Flavonoid or coumarin         |
| 5  | E       | -             | Blue                         | Flavonoid or coumarin         |

Figure 3. Profile chromatogram compound detection results. (I) Detection of isolates A, B, C, D, E with UV$_{254}$ (II) Detection of isolates A, B, C, D, E with UV$_{366}$ (III) Detection of isolates A, B, C, D, E with Cerium (IV) sulfate. Stationary phase: Silica gel 60 GF$_{254}$. Mobile phase: chloroform: ethyl acetate 3:1 (v/v)

The results of detection compound with reagent spray FeCl$_3$ against isolates of A, B, showed positive results (+) phenol compounds, but to isolate C, D, and E showed a negative result (-). Detection of compounds with reagent spray AlCl$_3$ followed UV$_{365}$ showed positive results (+) to isolate B (table 2), so that suspected isolates B contains flavonoids. According to Wagner [12] showed that the compounds detected by UV$_{365}$ blue fluorescence or blue-green indicate these compounds including flavonoids or coumarin in a class of phenolic. Isolates E also showed blue luminescence under UV$_{365}$ that can be suspected to contain coumarin compounds or flavonoids as well. But the predictions need to be further analyzed to determine the type of compounds.

Flavonoids and coumarin synthesized by two main pathways for biosynthesis of the aromatic ring, namely the shikimate pathway and acetate malonate. Coumarin is phenol compound derived from the shikimate pathway while flavonoid derived combination [13]. Coumarin showed a broad spectrum of
biological activities such as antibacterial activity, antifungal, anticoagulant, anti-inflammatory, antitumor and anti-HIV [14]. The other study revealed that a compound coumarin and xanthones have the ability to inhibit the growth of bacteria *Staphylococcus aureus* but not for bacteria *Escherichia coli*, *Actinobacter spp*, and *Crypto Coccus neofumas* [15, 16].

The flavonoids reported activity inhibit pathogenic agents in humans and is an antibiotic which can inhibit gram-positive and negative bacteria [17]. The other studies showed flavonoids contained in extracts of *P. macrocarpa* which have antimicrobial activity at concentrations of 0.3 mg/disk to 8 of the gram-negative bacteria and one of gram-positive bacteria such as *E. coli* [18]. It indicated that isolates was contained flavonoids or coumarin compounds are an antibacterial candidate, especially isolates of B. Detection of compounds must be strengthened with the identification using either UV-Vis spectrophotometry, IR, or NMR to obtain a pure chemical structure of the compound antibacterial candidates.

This study could be the basic for further research in the purification of isolates. It could known the specific compound that activity as an antibacterial to reduce the problem of decay fish or fish preservation. Fish is source of high protein, fat, vitamins, and minerals which are very good and prospective. According to Siswono [19], the main advantage of fish protein compared to other products is the completeness of the amino acid composition and simplicity to digest. The role of nutrition for healthy fish should be a good fish processing system with natural ingredients that are able to inhibit the activity of microbes or enzymes that improve the quality of fish as food.

4. Conclusion
The antibacterial activity of isolates (B, C, D, E) of the 70% ethyl acetate soluble portion had inhibitory activity. The inhibitory activity showed isolates B in *B. cereus* and isolates E in *P. aeruginosa* bacteria. The concentration of 20% ethyl acetate isolates soluble part based antibacterial activity test was enough optimum inhibit growth bacteria of *P. aeruginosa*, *B. cereus*, *E. coli*, *K. oxytoca*, and *E. aerogenes*. Test MIC and MBC of isolates B, C, D, and E in *B. cereus* and *P. aeruginosa*bacteria with concentration 20 % could not be determined.
References

[1] Wikandari P R, Suparmo, Y Marsono, dan E S Rahayu 2012 Jurnal Natur Indonesia 14(2): 120–125.
[2] Fardiaz S 1992 Mikrobiologi Pengelolaan Pangan Departemen Pendidikan dan Kebudayaan Direktorat Jendral Pendidikan Tinggi Pusat Antar Universitas Pangan dan Gizi Bogor: Institut Pertanian Bogor.
[3] Wulandari S, Sayuti, dan I Asmaini 2005 Jurnal Biogenesis 2(1): 30–35.
[4] Barus P 2009 Pemanfaatan bahan pengawet dan antioksidan alami pada industri bahan makanan Medan: Universitas Sumatera Utara.
[5] Collins C H, Patricia M Lyne, J M Grage 1989 Microbiological Methods 6th Edition London: Butterworth.
[6] Retmaningtyas E, E Purwani, dan T Purwoko 2009 Pemanfaatan Ekstrak Buah Mengkudu (Morinda citrifolia L) dan daun Pandan (Pandanus amaryllifolius Roxb.) sebagai Pengawet Alami Daging dan Ikan Segar LPPM UNS: Surakarta.
[7] Zakaria Z A, A M Desa, K Ramasamy, N Ahmat, A S Mohamad, D A Israf and M R Sulaiman 2010 Afr. J. Microbiol. Res. 4(1): 071-075.
[8] Jawetz E, J L Melnick, and E A Adelberg 2001 Mikrobiologi untuk Profesi Kesehatan (Review of Medical Microbiology) translated by H Tomang Jakarta: Penerbit EGC.
[9] Vinodh R, L K Elumalai, D Sangeetha, and R Rengasamy 2009 J. Biosci. Tech 1(1): 45-51.
[10] Liana I 2010 Aktivitas Antimikroba Fraksi dari Ekstrak Metanol Daun Senggani (Melastoma candidum D. Don) terhadap Staphylococcus aureus dan Salmonella typhimurium serta Profil Kromatografi Lapis Tipis Fraksi Teraktif Jurusan Biologi Fakultas Matematika dan Ilmu Pengetahuan Alam Universitas Sebelas Maret: Surakarta.
[11] Sunny A J 2011 Potensi Antibakteri Propolis Lebah Trigona spp Asal Bogor Departemen Biokimia Fakultas Matematika dan Ilmu Pengetahuan Alam IPB: Bogor.
[12] Wagner H 1983 Plant Drug Analysis Translation of the German Edition
[13] Kristanti A N, N S Aminah, M Tanjung, and B Kurniadi 2008 Fitokimia Jurusan Kimia FMIPA UNS: Surakarta.
[14] Dekic B R, N S Radulovic, V S Dekic, R D Vukicevic, and R M Palic 2010 Molecules 15: 2246-2256.
[15] Pattalung P N, Thongtheeraparwn P, Wiriyachitra P, and Taylor W C 1994 Planta Med. 60: 365–368.
[16] Verotta L, Lovaglio E, Vidari G, Finzi P V, Neri M G, Raimondi A, Parapini S, Tara melli D, Riva A, and Bombardelli E 2004 Phytochem. 2867-2879.
[17] Bylka W, I Matlawska, and N N Pilewski 2004 Review Article JANA 7 (2): 24-31.
[18] Hendra R, S Ahmad, M Y Shukor, and E Oskoueian 2011 Int. J. Mol. Sci. 12: 3422-3431.
[19] Siswono 2003 Ikan Air tawar kaya Protein dan Vitamin (http : gizi.net.com)