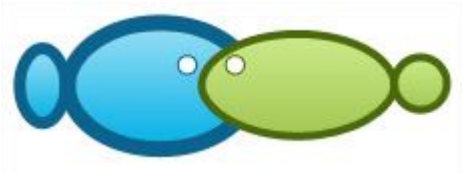

The potential of bangkal leaf (*Nauclea subdita* [Korth.] Steud.) extract as antibacterial in catfish *Pangasius hypophthalmus* culture

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Abstract. This study was aimed to evaluate the ability of bangkal leaf extract (*Nauclea subdita* [Korth.] Steud) as an antibacterial against *Aeromonas hydrophila* in *Pangasius hypophthalmus* (*in vitro* and *in vivo*). Bangkal leaves were extracted in methanol, ethyl acetate, and n-hexane as solvents. The extracts were assayed for coculture with *A. hydrophila*. The best fraction of the coculture assay was evaluated for minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and Scanning Electron Microscope (SEM). *In vivo* assay evaluated the survival rate of infected fish after administration of extracts and *A. hydrophila* colonies into the fish blood. The coculture assay showed that ethyl acetate fraction had the best ability to kill *A. hydrophila*. MIC assay of ethyl acetate fraction could inhibit at the minimum dose of 25 mg L^{-1}. MBC assay showed that *A. hydrophila* could not grow at 50 mg L^{-1} (2xMIC). Moreover, SEM analysis showed damage to *A. hydrophila* bacterial cells after administration of *N. subdita* ethyl acetate fraction of 500 mg L^{-1}. Survival rate appeared at the dose of 100 mg L^{-1}, in which the catfish mortality did not occur. The observation of bacterial colonies in the catfish blood showed no bacterial growth under the extract treatment of 100 and 150 mg L^{-1}. Therefore, ethyl acetate fraction of 100 mg L^{-1} was recommended to overcome infections caused by *A. hydrophila*.
Key Words: bacteria, coculture assay, MIC, MBC, SEM.

Introduction. Motile Aeromonad Septicemia (MAS), caused by *Aeromonas hydrophilla*, has become a serious problem in freshwater aquaculture. Several studies reported the presence of MAS outbreak in several species of freshwater fish, including *Anguilla anguilla* (Esteve et al 1993), *Clarias gariepinus* (Angka et al 1995), *Oreochromis niloticus* (Yambot 1998), *Carrasius auratus* (Maji et al 2006), *Pangasius hypophthalmus* (Olga et al 2006) and freshwater shrimp *Macrobrachium rosenbergii* (Abdolnabi et al 2015). The mortality rate of fish larvae infected by *A. hydrophilla* was reported up to 80-100% within 1-2 weeks (Cipriano 2001).

In general, antibiotics have been applied to control the disease, but long term improper use of antibiotics results in the occurence of multiresistance bacteria. Study conducted by Vivekanandhan et al (2002) found that there was *A. hydrophila* resistant to 15 antibiotics including methicillin and rifampicin (100%), bacitracin and novobiocin (99%), and chloramphenicol (3%). Resistance to β-lactam antibiotics is also detected in *A. hydrophilla* in Northern Portugal fish farm (Saavedra et al 2004). Furthermore, dissemination of antibiotics-resistanced species is observed as the result of horisontal resistance gene transfer (Schmidt et al 2001). Therefore, there is a necessity of finding other alternative treatment to control this MAS disease. Biological control using medical plants is a promising method for controlling pathogenic bacterial. Some medical plants have been evaluated for their ability as antibacterial against *A. hydrophila,* such as *Avicennia* sp. extract (Mulyani et al 2013), *Euphorbia hirta* (Salosso & Jasmanindar 2014), *Rhizophora mucronata* (Pradana et al 2014), *Impatiens balsamica* L. (Kusuma et al 2014), *Plumeria alba* (Ikrom et al 2014), *Andrographis paniculata* (Sinaga et al 2016).

Nauclea subdita ([Korth.] Steud.), Rubiaceae, is a tropical plant which grows in several different places, such as lowlands, swamps, streams, rivers and forests. In Indonesia, this species is well-known as a traditionally medical plant to treat fever, pain, dental caries, oral septic, malaria, dysentery, diarrhea, and central nervous system-related diseases, such as epilepsy (Abbah et al 2010). Generally, *Nauclea* sp. produces secondary metabolites, such as tannins, phenolics, steroids and alkaloids (Amos et al 2005; Ettebong et al 2014; Liew et al 2015). Several studies show that *Nauclea* sp. is a potential antimicrobial against several bacteria. Stem and bark of *N. subdita* are effective against pathogenic seawater *Vibrio parahaemoliticus* and *V. alginolyticus* (Jamaluddin et al 2015). Although the leaf of *N. subdita* is abundantly available, there is still no research to evaluate their potential as an antibacterial. Therefore, this study was aimed to evaluate the ability of *N. subdita* leaf extract in various solvents as antimicrobial against *A. hydrophila in vitro* and *in vivo* in *Pangasius hypophthalmus*.

Material and Method. This study was carried out for 5 months, January to May, 2019. Bangkal leaves (*N. subdita*) were collected from river bank of Bangkal village, Cempaka district, Banjabaru, South Kalimantan, and brought to the Laboratory of Organic Chemistry, Faculty of Basic Sciences, State Islamic University, Malang, East Java. Bacterial culture and antibacterial test were done in the Laboratory of Fish Disease and Health, Faculty of Fisheries and Marine Science, Brawijaya University.

Extraction of leaves of N. subdita. Leaves of *N. subdita* were cleaned, cut into small pieces and dried at 60ºC overnight. The dried leaves were blended to fine powder and extracted using a modified method based on Fatin et al (2012). One-hundred g of *N. subdita* powder was soaked in 400 mL of methanol, ethyl acetate and n-hexane, respectively, for 72 hours. The mixture was then separated using Whatman 42 filter paper. The extract obtained was evaporated by using vacuum rotary evaporator at 40ºC. The evaporation products were then used for antimicrobial testing.

Antimicrobial assay. This study employed bacterium *Aeromonas hydrophila* (ATCC 35654). This isolate was a collection of Fish Quarantine Station of Kupang, Indonesia. Antimicrobial assay was carried out using co-culture assay based on Fukuda et al (2008) and Moutinho et al (2017). Five mL of Tryptic Soy Broth (TSB) media were inoculated with 100 µL of overnight stock culture, then added with 500 µL of *N. subdita* extract of n-hexane, ethyl acetate and methanol, respectively. Bacterial culture with no extract was used as a control. The incubation process was conducted at 30°C for 24 hours. The product of co-culture assay was then performed in the plate of glutamate starch phenol (GSP) medium using total plate count (TPC) method. All treatments had 3 replications. The highest ability extract to inhibit *A. hydrophila* was then used for the next assay.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The minimum inhibitory concentration (MIC) test was conducted using double layer disc diffusion method (Duan et al 2006) in order to determine the inhibitory ability and the dose of the active ingredient resulting in the largest inhibition zone. MIC test used the modified method of Akhter et al (2008). The overnight culture of *A. hydrophila* (1×10^{10} cfu mL^{-1}) was placed into semi-solid Tryptic Soy Agar (TSA) medium at $\pm 40^{o}$C for 24 hours, then poured into a bacto agar medium. Disc papers with 30 µL of extract (concentrations between 12.5 mg L^{-1} to 1,000 mg L^{-1}), positive control (Gentamycin 120 mcg) and negative control (blank disk) were applied to the medium and incubated at 30^{o}C for 24 hours. The minimum bacterial concentration (MBC) was conducted by mixing the overnight culture of of *A. hydrophila* ($1x\ 10^{10}$ cfu mL^{-1}) with the extract of of *N. subdita* at the concentration of 0.5-16 times MIC for 24 hours. As much as 100 µL of mixed extract was cultured into *Pseudomonas Aeromonas selective Agar* (GSP) at $\pm 37^{o}$C for the next 24 hours to obtain the total bacterial count.

Scanning electron microscope (SEM) of bacteria. Vizualisation with SEM (FEI inscpect 550) was conducted to observe *A. hydrophila* after treatment with *N. subdita*

extract. Observations were performed after 12 and 24 hours of incubation and then compared with *A. hydrophila* without extract.

In vivo analysis. *In vivo* analysis of *N. subdita* potential was done on catfish *Pangasius* sp. of 21.22±0.2 g mean weight. The challenge test with *A. hydrophilla* (1.10^8 CFU mL^{-1}) was performed through immersion for 24 hours. The clinical signs of *A. hydrophilla* infection were observed after 24 hours. After that, treatment with ethyl acetate-extracted *N. subdita* was applied at various concentrations (50-150 mg L^{-1}) by immersion for 60 min. Treatment with no *N. subdita* extract was also taken as a control. The fish were then kept in the culture tanks (10# per 15 L aquarium). The fish survival rate and *A. hydrophila* count in the fish blood were calculated after 7 days of infection. The bacterial count in the fish blood was based on the method of Karam El-Din et al (2010).

Statistical analysis. To compare between treatments and control, one-way analysis of variance (ANOVA) was applied at the significance level of $p < 0.05$ using version 13.0 SPSS. Significant differences between treatments were revealed with Duncan's Multiple Range Test.

Results and Discussion

Antimicrobial assay. In this study, *N. subdita* was extracted using three different solvents, i.e. methanol, ethyl acetate and n-hexane. To reveal the effect of the *N. subdita* extract on the growth of *A. hydropila*, the co-culture assay was performed for 96 hours (Table 1).

Table 1

Total bacterial count of *A. hydrophila* cultured with *N. subdita* extracts with different solvents

No	Solvents	Total bacterial count (CFU mL^{-1}) (hour)			
		24 h	*48 h*	*72 h*	*96 h*
1	Methanol	0.00	0.00	$194x10^4$±0.08	$155x10^4$±0.10
2	Ethyl acetate	0.00	0.00	0.00	0.00
3	n-hexane	0.00	$29.5x10^6$±0.75	$20.02x10^{10}$±0.27	>$200x10^{10}$±0.00
4	Control	$20.3x10^9$±0.7	$23.1x10^{10}$±0.51	>$200x10^{10}$±0.00	>$200x10^{10}$±0.00

Note: too numerous to count (tntc) = > 200 colonies per filter to be scored (Haas & Heller 1988).

Table 1 demonstrates that after 24 hours of incubation, all *A. hydrophila* cultured with addition of *N. subdita* extracted in various solvents showed no growth. In contrast, the bacterial culture without extracts (control treatment) showed significant growth up to 2.03 $x10^{10}$ CFU mL^{-1}. In the next 24 hours, the bacterial flask with addition of n-hexane-extracted *N. subdita* showed a significant growth (2.95 $x10^7$ CFU mL^{-1}) and continued to increase until too numerous to count at 96 h. Similar situation was also observed in *A. hydrophilla* culture with addition of methanol-extracted *N. subdita* after 72 hours. No growth was only detected in the bacteria cultured with addition of ethyl acetate-extracted *N. subdita* even after 96 hours of culture (Table 1). The antibacterial activity suggests that ethyl acetate-extraced *N. subdita* contains the active phytochemical responsible for bacterial elimination. Antibacterial activities have been documented in the genus of *Nuclea* such as *N. latifolia* (Okwori et al 2008; Okechukwu et al 2015), *N. pobeguinii* (Seukep et al 2016) and *N. obversiffolia* (Khan et al 2001).

MIC and MBC. MIC is the lowest concentration of antimicrobial compound to prevent the bacterial growth. The results of MIC assay are presented in Table 2.

As a complementary of MIC, the MBC was determined using *in vivo* test. The MBC test showed that there was bacterial growth at a concentration of 0.5 MIC or 12.5 mg L^{-1} (Table 3). The same finding as MIC was also confirmed in MBC that the minimum concentration to inhibit *A. hydrophila* was 25 mg L^{-1} after 24 hours. In addition, *A. hydrophyla* population was detected after 48 hours. No growth of *A. hydrophilla* was observed after 24 and 48 hours with the addition of *N. subdita* extract at the minimum

concentration of 50 mg L^{-1} (2 x MIC). Therefore, the MBC value was 50 mg L^{-1} and it could be used for the minimum value of treatment for the in vivo study.

Table 2

Minimum inhibitory concentration (MIC) testing

Ethyl acetate-extracted N. subdita (mg L^{-1})	*Diameter of inhibition zone (mm)*	
	24 h	*48 h*
12.5	0.00±0.00	0.00±0.00
25	3.66±0.57	1.66±0.57
50	5.33±0.57	4.00±0.00
100	8.66±0.57	8.66±0.57
200	9.66±0.57	9.66±0.57
300	10.00±0.00	10.00±0.00
400	14.00±0.00	14.00±0.00
500	14.66±0.57	14.66±0.57
600	15.00±0.00	15.00±0.00
700	15.33±0.57	15.33±0.57
800	15.66±0.57	15.66±0.57
900	16.00±0.00	16.00±0.00
1000	17.66±0.57	17.66±0.57

Table 3

MBC test results on GSP agar

Concentration	*Concentration (mg L^{-1})*	*Bacterial count (CFU mL^{-1})*	
		24 hours	*48 hours*
Control	0.0	tntc	tntc
0.5 MIC	12.5	tntc	tntc
1 MIC	25.0	0.0	$3.7x10^2$±0.15
2 MIC	50.0	0.0	0.0
4 MIC	100.0	0.0	0.0
8 MIC	200.0	0.0	0.0
16 MIC	400.0	0.0	0.0

tntc = too numerous to count.

Some active compounds from plants that can damage bacterial cell walls are phenols, flavonoids, saponins, alkaloid, terpenoids and tannins (Fatin et al 2012; Jamaluddin et al 2015; Li et al 2017). Each group of the compounds has different ability to inhibit the bacterial growth. It could result from different bioactive substances contained that have different synergistic effects as well, depending on the characteristics and the morphology of the bacteria. Phenolic compounds are classified into four main groups, flavonoids, phenolic acids, lignans, and tannin polymers (Nohynek et al 2006). Polyphenol and flavonoid compounds belong to phenol group (Kaur & Arora 2009). The antibacterial mechanism of flavonoid is to inhibit the synthesis of nucleic acids, cytoplasmic membrane function, energy for metabolism, attachment and biofilm formation, porins as cell membrane compiler, change membrane permeability, and weaken pathogenicity (Xie et al 2015). Flavonoids could inhibit bacterial growth by damaging cell walls, deactivating enzymes, binding to adhesin, and damaging cell membranes (Cowan 1999).

According to Sabbineni (2016), non-polar materials work on the outer membrane of bacterial cell walls. Super-molecular parts can enter and disrupt the membrane structure. The bacterial cell walls affect permeability and variation in response to hydrophilic antibacterial compounds. In the cytoplasmic membrane, active phenol molecules can penetrate the membrane and bind to membrane proteins. The active phenol molecule acts on the cytoplasm and nucleus of bacterial spores. Besides that, active phenol molecule acts as an active disinfectant and oxidizer. This causes damage to the core structure in the spores.

Liew et al (2014) reported nine monoterpenoid indole alkaloids in *N. subdita*. Alkaloid antibacterial with purified enzymes showed that indolizidine alkaloids could

inhibit dihydrofolate reductase. Isoquinoline alkaloids also inhibit type I topoisomerase (Cushnie et al 2014). The effects of alkaloids on virulence gene regulation systems were like quorum sensing and virulence factors, such as sortase, adhesin, and secretion systems. Alkaloids from *C. citrinus* show antibacterial activity and inhibit transport of ATP-dependent compounds across cell membranes (Mabhiza et al 2016). Terpenoid compounds worked by damaging bacterial cell membranes (Cowan 1999). The terpenoid compounds react with the active site of the membrane, dissolve the lipid constituents, and increase their permeability. Terpenoid phytadiene and 1,2-seco-cladiellan from the meniran herb (*Phyllanthus niruri*) were reported to be active against *Staphylococcus aureus* and *Escherichia coli* (Gunawan et al 2018). According to Daisy et al (2008), terpenoid compounds 6-[1- (1,13-dimethyl-4,5,8,9,10,11,12,13,14,15,16,17 dodekahidro-1H-syclopenta [alpa] phenan tren-17-il)] - 3-methyl-3,6-dihydro-2H-piranon isolated from *Elephantopus scaber* can inhibit the activity of the autolysin enzyme in *S. aureus* with strong interactions on the active site of the enzyme residue. Some terpenoid components (eucalyptol, α-terpineol and linalool) from essensial oil causes the alteration of outer membrane, change of the function of cell membrane and intracellular material leakage (Zengin & Baysal 2014). Lv et al (2011) also reported that terpenoid from commercial essensial oil could affected the cell membrane interity. Some finding shows that the monoterpen derived from drugs results in membrane permeability and intracellular material leakage alteration (Trombetta et al 2005).

Akiyama et al (2001) explained the antimicrobial mechanism of tannins that could induce the complexation with enzymes or substrates and inactivate microbial cell adhesin. Tanic acid can work like siderophore to chelate iron from the medium, so that iron was not available for microorganisms. Payne et al (2013) reported that tannic acid inhibits the formation of *S. aureus* biofilms through a mechanism that relied on putative transglycosylase IsaA. Overexpression of wild-typed IsaA inhibits the formation of biofilms, while overexpression of IsaA that died catalytically does not affect.

Saponins have detergent-like properties and can increase the permeability of bacterial cell membranes without damaging them. Saponins, when used with antibiotics, can facilitate the entry of antibiotics through bacterial cell wall membranes (Arabski et al 2012). Saponin is concentrated in the smooth and essential part of the cell membrane. Saponins are potent surface tension-reducing compounds and work as antimicrobials. Saponins can interfere with the stability of bacterial cell membranes that causes bacterial cells to undergo lysis. Saponin is also known as a natural antibacterial and energy booster and beneficial in reducing inflammation in the upper respiratory system (Ogbuagu 2008).

A. hydrophila morphology after addition of N. subdita extract. Observation of *A. hydrophila* morphology after addition of *N. subdita* extract was conducted using scanning electron microscopy (SEM). The morphological changes of *A. hydrophilla* were observed after 12 and 24 hour addition of *N. subdita* extract as depicted in Figure 1.

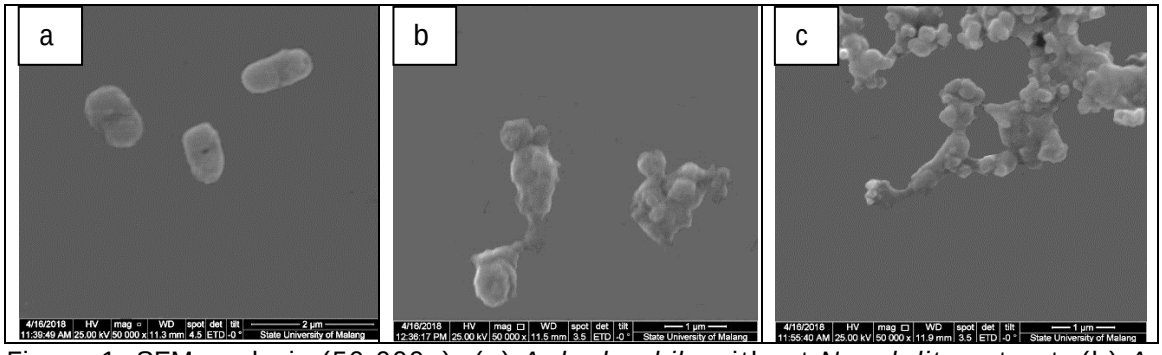

Figure 1. SEM analysis (50,000x). (a) *A. hydrophila* without *N. subdita* extract, (b) *A. hydrophila* with addition of ethyl acetate- extracted *N. subdita* after 12 hours, and (c) *A. hydrophila* with addition of ethyl acetate-extracted *N. subdita* after 12 hours.

SEM analysis of *A. hydrophila* without *N. subdita* extract (Figure 1a) showed that the bacterial cells were in the normal condition without any damage. After addition of *N. subdita* extract for 12 hours, the damage of cell wall was observed (Figure 1b). For the next 24 hours, the severe cell damage occurred and formed some debris (Figure 1c). Meng et al (2016) also found the irregular deformities of *Klebsiella pneumoniae* after 4, 8 and 24 hour treatment with essential oil from *Juniperus rigida* leaves. The bioactive compounds in *N. subdita* extract reacted with the cell membrane of *A. hydrophila* and caused the disintegrity of the cell membrane (Figure 1b and 1c). The role of antimicrobial compound is to inhibit the cell wall synthesis and its functions as well as disruption of cell membrane integrity. The cell membrane acts as a barrier with selective permiability, active transport function and arrangement control in the cells (Nguyen et al 2011). If the integrity of the cell membrane is disrupted, macromolecules and ions will be released from the cell and the cell damage or death would be occured.

In vivo study. *In vivo* study was conducted in *Pangsius* sp. to reveal the potency of *N. subdita* as antibacterial agent. After infected with *A. hydrophila* at the lethal concentration (10^8 cells mL^{-1}), *Pangasius* sp. was treated with *N. subdita* extract with immersion method at various concentrations. After 7 days, the survival rate of *Pangasius* sp. was observed as those presented in Figure 2.

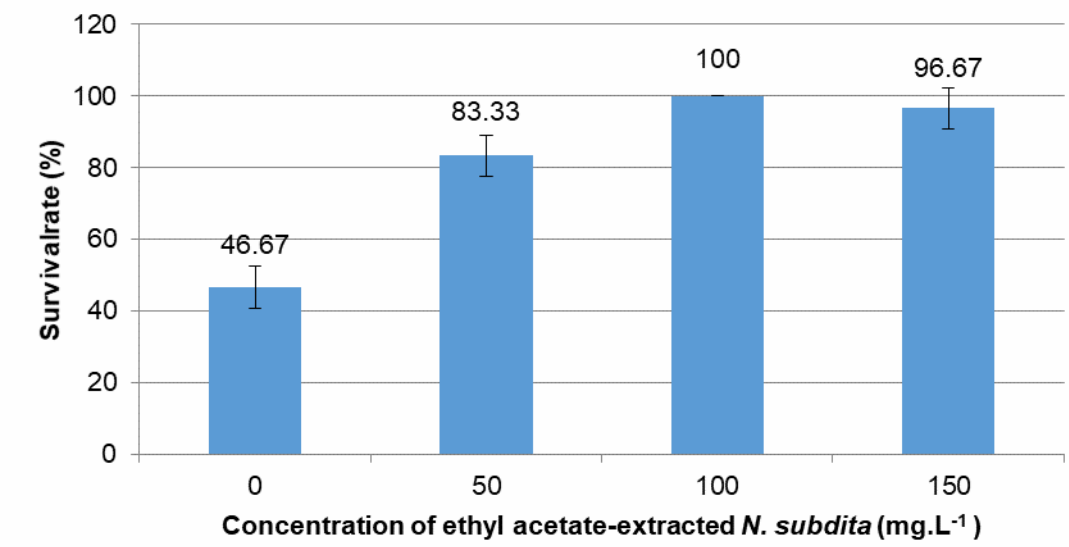

Figure 2. Survival rate of *Pangasius hypophthalmus* treated with ethyl acetate extracted *N. subdita*.

Statistical analysis using ANOVA revealed that treatment with *N. subdita* extract gave a significant effect on the survival rate (SR) of *P. hypophthalmus*. ($p < 0.05$). The highest SR was observed with the treatment of *N. subdita* extract at the concentration of 100 mg L^{-1}. This treatment increased the fish SR up to more than two times that without *N. subdita* extract (Figure 2). Increase in *N. subdita* concentration above 100 mg mL^{-1} gave similar effect on SR of *P. hypophthalmus*. This increase could be due to the active ingredients in *N. subdita* extract.

A. hydrophila count in Pangasius sp. blood. The use of *N. subdita* extract as an antibacterial agent can be confirmed through its ability to inhibit the proliferation of *A. hydrophila* in *Pangasius* blood. The *A. hydrophila* counts in the blood of *Pangasius* after infection and treatment are presented in Table 4.

Table 4 shows no *A. hydrophilla* detected in *Pangasius* blood at the begining of study. The challenge assay through immersion method in 1 x 10^8 cfu mL^{-1} of *A. hydrophilla* resulted in the presence of these bacteria in the blood at various concentration from $2.7x10^6$ to $5.4x10^6$. Seven (7) days after immersion in *N. subdita* extract, bacterial decrease were observed in all treatments. The decrease of *A. hydrophila* population in treatment with no *N. subdita* extract implied the presence of

immune response in *Pangasius* sp. The treatment with 50 mg L^{-1} of *N. subdita* extract revealed that the decline of *A. hydrophilla* in *Pangasius* blood was significant up to 99%. With higher concentration of *N. subdita* extract, there was no more *A. hydrophilla* population in *Pangasius* blood. The significant decline in *A. hydrophilla* population in *Pangasius* blood showed that the active compund in *N. subdita* extract was potential to be antimicrobial agent.

Table 4

Total bacterial count of *A. hydrophila* in *Pangasius* sp. blood during study

Time	*Total A. hydrophila count at various concentrations of N. subdita (cfu mL^{-1})*				
	0	*50*	*100*	*150*	*Control (no infection)*
Initial	0	0	0	0	0
After infection	$5.4x10^6$ ±0.62	$2.7x10^6$ ±0.62	$3.03x10^6$ ±0.15	$3.47x10^6$ ±0.42	0
7 days after immersion in *N. subdita* extract	$3.77x10^5$ ±0.31	$5.7x10^2$ ±0.46	0	0	0

Conclusions. Ethyl acetate-extracted *N. subdita* was found to be a potential antimicrobial agent against *A. hydrophilla in vitro* with MBC value of 50 mg L^{-1}. *In vivo* study showed that immersion in ethyl acetate extracted *N. subdita* reduced the population of *A. hydrophilla* in the fish blood up to 100 % and increased the survival rate of *Pangasius* sp. Application of 100 mg L^{-1} of ethyl acetate-extracted *N. subdita* gave the highest survivorship with no mortality of *Pangasius* sp. after challenge.

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