

***Sonneratia alba* Extract Protects the Post Larvae of Tiger Shrimp *Penaeus monodon* against *Vibrio harveyi* and *Saprolegnia* sp.**

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Abstract. The research aimed to study *Sonneratia alba* leaves extract to inhibit *Vibrio harveyi* and *Saprolegnia* sp., and to evaluate its effectiveness to protect the post larvae of tiger shrimp against the infections. The leaves of *S. alba* were cleaned, chopped, dried, macerated and extracted in three different solvents namely ethanol 80%, freshwater and saline water. The post larvae 8 then immersed with the extract and infected with *Vibrio harveyi* and *Saprolegnia* sp. The clinical signs, pathological anatomy, total vibrio count, prevalence, survival rate, and relative percentage of survival were observed. The results showed that *S. alba* leaves extract inhibited *V. harveyi* and *Saprolegnia* sp. on post larvae of tiger shrimp, improve survival rate and relative percentage survival. The most effective dosage was 1,250 mg/L either for ethanolic, freshwater and saline water solvents.

1 Introduction

The disease outbreak in tiger shrimp culture in East Kalimantan province, Indonesia is still struggling to be solved. Shrimp mortality occurred in the larvae stage in hatchery to post larvae stage in grow out ponds, and even just before harvesting. The common cause of mortality is *Vibrio harveyi* and in larvae stadia can also caused by fungi [1]. The main disadvantage in aquaculture is the sudden outbreak of the disease, mainly due to *Vibrio* spp, which is considered a significant problem for the development of this sector with severe economic losses worldwide [2]. Disease prevention are still not resolved properly due to usage of chemicals and antibiotics. The use of these chemicals are often uncontrolled and leads to bacterial resistance and toxicity in shrimp [3]. Several studies have shown that some plant extracts active against fungi and bacteria both in vitro and in vivo. [3, 4, 5, 6, 7, 8].

Mangrove grows well in coastal areas of East Kalimantan, Indonesia. Mangrove is widely used for household needs, as well as food, beverages and traditional medicine. Mangrove is a source of several bioactive compounds. Mangrove plants are used as medicines by the community and the extracts of it known to inhibit the growth of pathogenic microorganisms [9, 10]. Research in mangrove as a medicine has been widely practiced, as well as mangrove screening as an antibacterial against disease in shrimp [1, 4, 5, 11, 12].

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Mangrove is rich in bioactive compounds [13]. Mangroves can be used as a new bioactive source of potential natural products used to control microbial disorders [14]. The mangrove phytochemical analysis has revealed important chemicals such as saponins, alkaloids, glycosides, tannins, steroids, flavonoids, gums, phytosterols, and reducing sugars [15]. Extracts of methanol bark of *S. alba* and *A. marina* fruit showed a 15.00 mm inhibition zone to *Salmonella typhi*, its acetic acid extract showed a 14.00 mm inhibit zone against *Listeria monocytogenes* [16]. Extract of *S. alba* can inhibit *V. harveyi* and *Saprolegnia* sp. [17]

Mangroves are widely found in Indonesia but the utilization of bioactive materials has not been developed yet [18]. This research aimed to study *Sonneratia alba* leaf extract to inhibit the growth of *V. harveyi* and *Saprolegnia* sp., and to improve the survival of tiger shrimp.

2 Methods

2.1 *Sonneratia alba* Extraction

Leaf of *S. alba* was collected from the coast in Muara Badak District, Kutai Kartanegara Regency, East Kalimantan. The leaves were cleaned, and drained at room temperature without direct sunlight exposure. After drying, the samples were chopped and macerated with three different solvents, i.e. 80% ethanol, freshwater and saline water at a salinity of 20 ‰ for 24 hours. After maceration, each solvent was evaporated by rotary evaporator until the remaining solvent around 30% of the initial solvent volume. For aqueous extract, the evaporation was carried out until the remaining solvent was around 10% of the initial volume [17].

2.2 Microbial Preparation

V. harveyi and *Saprolegnia* sp. were used for the infected test. They are obtained from the Laboratory of Aquatic Microbiology Faculty of Fisheries and Marine Sciences Mulawarman University. *V. harveyi* and *Saprolegnia* sp. were tested for pathogenicity by injecting to ten shrimps with individual body weight of 6 g. The injection was carried out intramuscularly 0.1 mL with at bacterial dilution of 10^3 . Three days post injection, the shrimp showed a reddish on the body. *V. harveyi* then reisolated from hepatopancreas and reinfected to the shrimp. The procedure was repeated three times. Finally, *V. harveyi* was isolated and cultured on Thiosulfate Citrate Bile Salt Sucrose agar (TCBSA) medium and incubated for 24 hours at 30°C. *V. harveyi* was cultured in Tryptic Soy Broth (TSB) with 2% NaCl. The *Saprolegnia* sp. Was cultured on Potato Dextro Agar (PDA) medium and incubated at 33 °C for 48 hours.

2.3 Post Larvae Shrimp

Post larvae (PL) 8 was obtained from the hatchery of Windu Permata in Muara Badak of East Kalimantan. Post larvae was antibiotic-free, also free from *Vibrio* sp. by bacterial testing on TCBSA medium. The shrimp was acclimatized for 1 day in the aquarium at a density of 20 shrimp.

2.4 The Treatment

Sterile saline water (free from *Vibrio* sp. and *Saprolegnia* sp.) was used in this research. All equipment used in this research were disinfected and dried. The water salinity was 23 ppt, and free of *V. harveyi*, *Aeromonas* sp., *Pseudomonas* sp., and *Saprolegnia* sp. The aquarium

and other facilities were washed with soap and soaked with potassium permanganate (KMNO₄). Total volume of water in each aquarium was 5 litres.

The treatments were ethanol, water and sea water extract of *S. alba*. Each *S. alba* extract which had concentrations of 750, 1,000, and 1,250 ppm. Control treatments consisted of antibiotic oxytetracycline as a positive control and PBS 0,85% as a negative control. The shrimps were soaked in each aquarium that were filled with each treatment and replicated three times.

The infected test with each of *V. harveyi* and *Saprolegnia* sp. was performed after 48 hours of treatment by soaking shrimp in 1 mL/litre with at microbial dilution of 10⁶ into each aquarium. The shrimp was reared for 21 days.

2.5 Data Analysis

This study used a complete randomized design with three replications. The data was analyzed using analysis of variance test, then followed by Duncan test if there were significant differences. The clinical symptoms of shrimp were observed and analyzed every day by calculating the percentage of inactive (passive) behavior, decreased appetite, weakened reflexes, reddish and incomplete of body. Anatomical pathology observation, such as damage and changes of the head, legs and tail, was performed on dead shrimp and at the end of the study. The Anatomical pathology were analyzed descriptively. In addition, at the day 14 and 21 of the study, the total bacterial content of *V. harveyi* of shrimp and water samples were enumerated by total count or total vibrio count (TPC/TVC). The prevalence was obtained from the percentage of shrimp infected with *V. harveyi* or *Saprolegnia* sp., that have reddish on the body and positive isolation. The survival rate was calculated by percentage of live shrimp from each treatment, while Relative Percentage of Survival (RPS) was calculated by the formula: $RPS = 1 - \frac{(\text{prawn treatment mortality})}{(\text{control prawn mortality})} \times 100\%$ [1]

3 Result and Discuss

3.1 Clinical Symptom

The condition of the shrimp is active and healthy after being treated with the extract of *S. alba*, however the body of the shrimp turned slightly bluish. This was due to the absorption of extract into the shrimp's body, which was the shrimp response. The shrimp did immune response to the extract that enters its body, it was the chromatophore reaction. The color changing of shrimp indicated the occurrence of body reaction toward the extract treatment, and it was caused by the enlargement of shrimps' cuticle [4, 11]. Post larva of shrimp appeared normal, healthy, active and did not cause any mortality and specific clinical symptoms after 48 hours of treatment.

The clinical symptoms were observed at 5 and 10 days after given extract of *S. alba*. Shrimp shows weakness and appetite decreases after being infected by *V. harveyi* and *Saprolegnia*, the clinical symptoms even more severe on negative control, such as weakness, decreased appetite, slow and weak reflexes, and reddish body. The overall clinical symptoms were in Table 1. *Sonneratia alba* extract was able to suppress *V. harveyi* and *Saprolegnia* sp. attack, so the clinical symptoms percentage of *S. alba* extract was lower than the controls. The extract of mangroves have the potential as antifungal and antibacterial activity, as well as gastric antioxidants, and able to heal wounds [19].

Table 1. The clinical symptoms of tiger shrimps which were treated *S. alba* extract

	Day	Clinical symptoms of shrimps which were infected with <i>V. harveyi</i> (%)										
		Extract of <i>S. alba</i> in ethanol (ppm)			Extract of <i>S. Alba</i> in freshwater (ppm)			Extract of <i>S. alba</i> in saline water (ppm)			Control +	Control -
		1,250	1,000	750	1,250	1,000	7,50	1,250	1,000	750		
inactive	5	0.00	0.00	15.00	0.00	15.00	20.00	0.00	0.00	25.00	10.00	30.00
	10	0.00	5.00	15.00	10.00	15.00	10.00	0.00	5.00	10.00	20.00	20.00
weakened reflexes	5	0.00	0.00	10.00	0.00	10.00	25.00	0.00	0.00	20.00	20.00	40.00
	10	0.00	0.00	5.00	0.00	0.00	10.00	0.00	10.00	15.00	15.00	40.00
decreased appetite	5	5.00	0.00	20.00	0.00	0.00	25.00	0.00	0.00	20.00	10.00	60.00
	10	0.00	0.00	15.00	0.00	0.00	20.00	0.00	0.00	15.00	20.00	50.00
reddish body	5	0.00	0.00	0.00	0.00	0.00	10.00	0.00	0.00	10.00	15.00	30.00
	10	0.00	0.00	20.00	5.00	5.00	15.00	5.00	15.00	20.00	20.00	50.00
Incomplete of body	5	0.00	0.00	0.00	0.00	0.00	10.00	0.00	10.00	10.00	20.00	20.00
	10	0.00	0.00	5.00	0.00	0.00	10.00	0.00	5.00	15.00	15.00	40.00
	Day	Clinical symptoms of shrimps which were infected with <i>Saprolegnia</i> sp. (%)										
		Extract of <i>S. alba</i> in ethanol (ppm)			Extract of <i>S. Alba</i> in freshwater (ppm)			Extract of <i>S. alba</i> in saline water (ppm)			Control +	Control -
		1,250	1,000	750	1,250	1,000	750	1,250	1,000	750		
inactive	5	0.00	0.00	10.00	0.00	20.00	20.00	0.00	0.00	15.00	0.00	40.00
	10	0.00	5.00	5.00	0.00	0.00	5.00	0.00	0.00	10.00	15.00	60.00
weakened reflexes	5	0.00	0.00	10.00	0.00	10.00	15.00	0.00	10.00	5.00	30.00	90.00
	10	0.00	0.00	10.00	0.00	0.00	5.00	0.00	0.00	10.00	20.00	80.00
decreased appetite	5	0.00	0.00	10.00	0.00	0.00	20.00	0.00	0.00	15.00	40.00	60.00
	10	5.00	10.00	5.00	0.00	0.00	0.00	0.00	0.00	0.00	20.00	65.00
reddish body	5	0.00	0.00	0.00	0.00	10.00	15.00	0.00	0.00	0.00	20.00	50.00
	10	0.00	0.00	5.00	0.00	0.00	10.00	0.00	10.00	15.00	20.00	60.00
Incomplete of body	5	0.00	0.00	5.00	5.00	10.00	15.00	0.00	10.00	10.00	20.00	40.00
	10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.00	15.00	15.00	40.00

3.2 Anatomical Pathology

Better anatomical pathology was shown by shrimp in all extract treatment when compared to controls. The tails and legs of some shrimps that treated with 750 ppm water extract of *S. alba* became reddish, as well as the tails of some shrimp with 750 ppm ethanol extract and saline water of *S. Alba*. The Anatomical pathology of shrimp on the negative control showed symptoms of *vibrio* attack, which was the tail, legs, gills and body redness. Some shrimp were deformed and failed moulting, this condition caused mortality. The anatomical

pathology of shrimp on negative control that was infected with *Saprolegnia* showed dirty feet and body, while the shrimp were treated the extract showed normal reaction.

Vibrio sp. caused dirty gills, changing the color to yellow or pale red, dark and in the carapace, abdominal, and tail, also dark slightly redness hepatopancreas [20]. The shrimp were attacked by *vibrio* showed changing appearance symptoms like changing color to reddish black, red patches on the legs and tail, hemorrhage on the body, deformity and molting failure [11].

3.3 Total Vibrio Count

V. harveyi was isolated from shrimp and water media. The Total Vibrio Count (TVC) showed that the lowest measurements was in 1,250 ppm ethanol extract of *S. alba* treatment, followed by the treatment of 1,250 ppm saline water extract of *S. alba* and control positive, then 1,000 ppm ethanol extract of *S. alba* (Fig. 1). The ethanol, freshwater and saline water extracts of *S. alba* were able to suppress the attack of *V. harveyi* and *Saprolegnia* sp. in shrimp.

The ability of *S. alba* extract as an antimicrobial was comparable to antibiotics. The methanol extract of *S. alba* bark showed a 15 mm inhibition zone against *Salmonella typhi*. Acetone extract of *S. alba* leaves showed a 14 mm inhibition zone against *Listeria monocytogenes* [16]. The inhibition zone of *S. alba* ethanol extract 1,000 ppm to *V. harveyi* is 12.67 mm, and *Saprolegnia* is 12.00 mm, and Minimal inhibitory concentration (MIC) of *S. alba* to *V. harveyi* is 3.91 µg mL⁻¹, and MIC against *Saprolegnia* sp. is 7.81 µg mL⁻¹ [1]. MIC of mangrove plants against pathogenic bacteria ranged from 20-640 mg/mL [21]. Methanol extracts of *Sonneratia caseolaris* was effective on all gram negative bacteria [9].

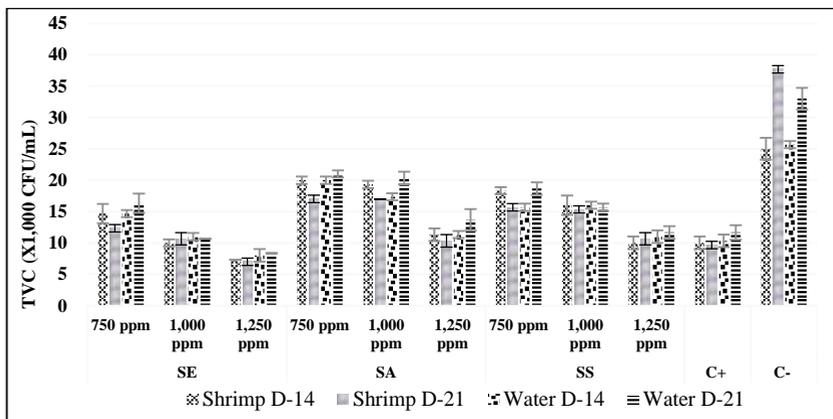


Fig. 1. The Total Vibrio Count (TVC) on the tiger shrimp and water media; Note: SE= *S. alba* in ethanol; SW= *S. Alba* in freshwater; SS= *S. Alba* in saline water; C+= positive control; C-= negative control.

3.4 Prevalence

The prevalence infected of the *V. harveyi* and *Saprolegnia* sp. in shrimps that were treated with the extract *S. alba* leaves, showed that bioactive of *S. alba* could reduce the prevalence of microbial infected (Fig. 2). The average prevalence attack of *V. harveyi* and *Saprolegnia* sp. on extract treatment was 12.67-56.67 %, and 2.33-47.33%. The average prevalence attack of *V. harveyi* and *Saprolegnia* sp. on the negative control was 75.00-81.33 %, and 69.33-72.33%.

Sonneratia caseolaris bark has the potential as a stable antimicrobial and antioxidant and can be used as an antimicrobial and natural antioxidant agent in the clinical, pharmaceutical and food processing industries [22]. Mangrove plants can be utilized as a source of natural antifungal drugs[23].

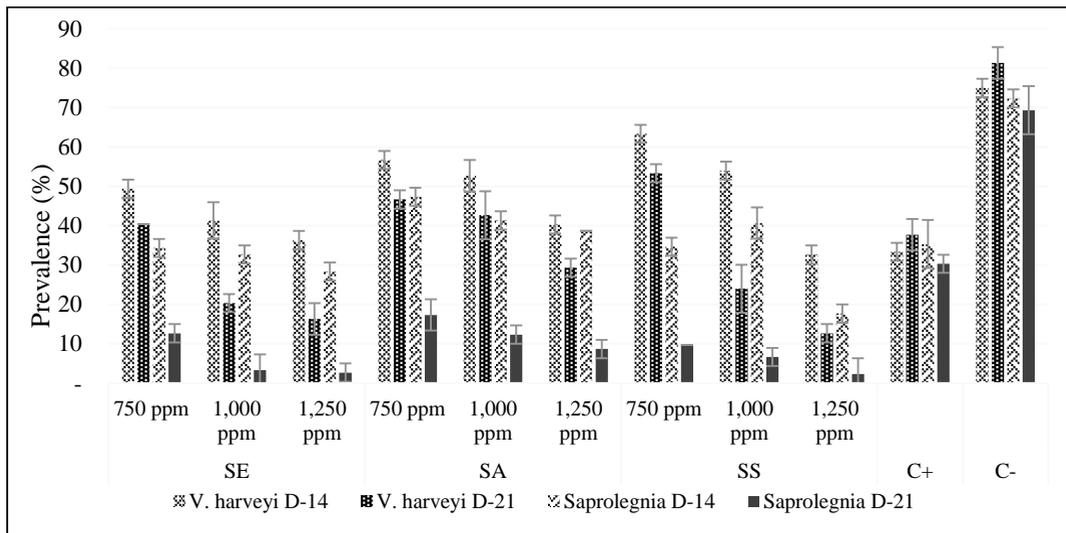


Fig. 2. The prevalence on the tiger shrimp; Note: SE= *S. alba* ethanol extract; SW= *S. Alba* freshwater extract; SS= *S. Alba* saline water extract; C+= positive control; C-= negative control

3.5. Survival Rate

The isolation of dead shrimp showed positive for vibrio or saprolegnia infection. The ethanol, freshwater, and saline water extract of *S. alba* leaves can inhibit *V. harveyi* and *Saprolegnia* sp. on shrimp. Survival rate on shrimp that were treated with ethanol extract of *S. alba* leaves and infected with *V. harveyi* and *Saprolegnia* sp. ranged from 58.33-80.67% and 54.33-78.33%, water extract of *S. alba* 46.67-73.33% and 41.33-69.33%, sea water extract of *S. alba* 57.00-81.33% and 54.67-82.67%, while positive control 73.33 and 65.33% and negative control 35.00 and 37.33% as shown in Fig. 3.

S. Alba extracted in saline water and ethanol with concentration 1250 ppm resulted the highest survival rate against *V. harveyi* compared to positive control, followed by 1,250 ppm of *S. alba* in freshwater solvent. The highest survival rate of shrimp which were infected with *Saprolegnia* sp. was also in the treatment of 1,250 ppm *S. Alba* in saline water solvent followed by 1,250 ppm in ethanol and 1,250 ppm in freshwater and control positive.

Several researchers around the world have found evidence that mangrove extract has great potential against pathogenic microbes. Mangrove extract using ethanol solvent, freshwater and saline water inhibit the growth of various microbes by in vitro test [17]. Antimicrobial compounds of the plant have therapeutic potential because natural ingredients are functionally work without side effects which often associated with synthetic antimicrobials [24].

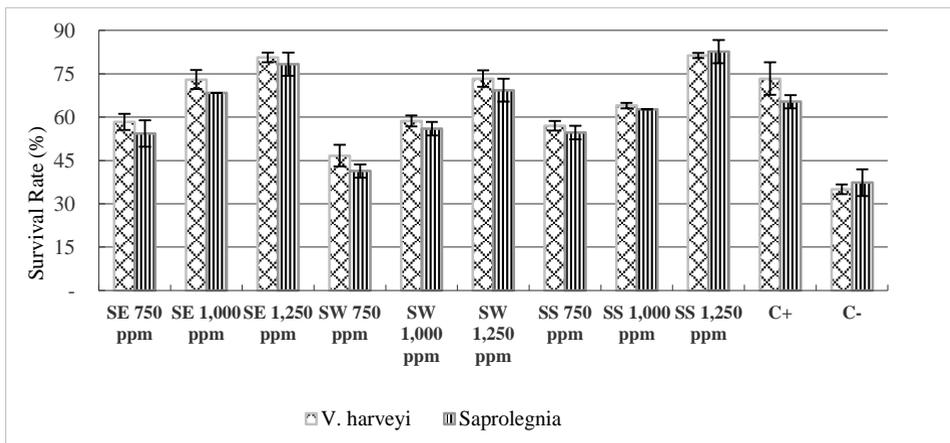


Fig. 3. The Survival Rate of tiger shrimp; Note SE= *S. alba* in ethanol; SW= *S. Alba* in freshwater; SS= *S. Alba* in saline water; C+= positive control; C-= negative control

3.6 Relative Percentage of Survival

The ability of *S. alba* extracts 1,250 ppm to protect the post larvae of tiger shrimp against *V. harveyi* attacks compared to antibiotic were not different. The second best treatment is 1,000 ppm in ethanol and 1,000 ppm in saline water. The ability of *S. alba* extracts protects against *Saprolegnia* sp. the best is also on saline water solvent of *S. alba* 1,250 ppm leaf which is different from antibiotic treatment but not different from freshwater and ethanol solvent treatment. The second best is 1,250 ppm in ethanol, followed by 1,250 ppm in water (Fig. 4).

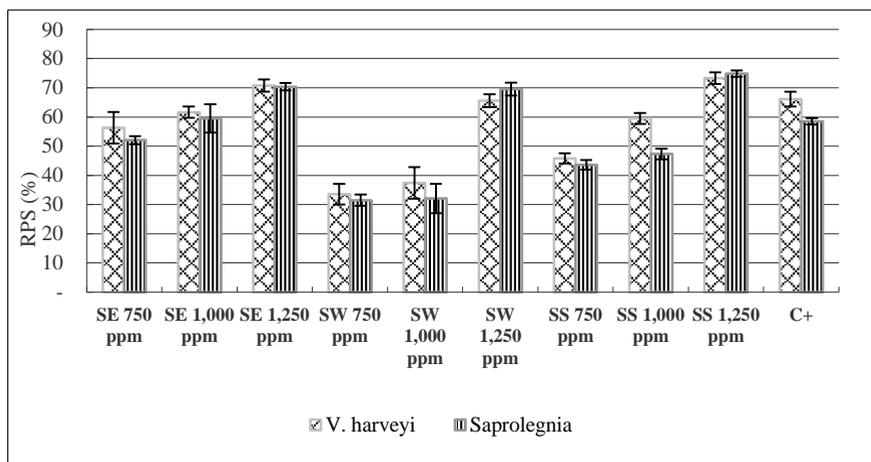


Fig. 4. The Relative Percentage of Survival (RPS) of tiger shrimp; Note: SE= *S. alba* in ethanol; SW= *S. Alba* in freshwater; SS= *S. Alba* in saline water; C+= positive control; C-= negative control

Sonneratia alba leaf extract has the ability to inhibit *V. harveyi* and *Saprolegnia* sp. infection and improve survival of post larvae of tiger shrimp. The ability to inhibit these microbes can be seen from the results of TVC test, which showed the content of TVC on shrimp that was treated with extracts have low values, especially on ethanol extract and sea water extract. *S. alba* leaf extract is able to protect against microbial attacks. The ability of *S. alba* leaf extract indicates that this plant contains antimicrobial ingredients. The mangrove plant is considered

an important source for chemical components of medicinal materials. Bioactive of mangrove can be used as antioxidants, anti-cancer and component drugs [25, 26]. Mangrove plants are a good source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins [24].

4 Conclusion

Sonneratia alba leaf extract is potentially to be used as an antimicrobial. These plants are able to inhibit *V. harveyi* and *Saprolegnia* sp., reduce prevalence attack of microbial, improve survival rate and relative percentage of survival post larvae of tiger shrimp.

5 Acknowledgement

This research is part of Superior Research of Higher Education funded by Directorate of Research and Community Service, Directorate General of Research and Development, Ministry of Research, Technology and Higher Education in 2018-2019. We would like to thank to Directorate of Research and Community Service, Rector and Head of Institution Mulawarman University Research.

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