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Potency of the *Piper betle* and *Ocimum basilicum* as a natural antibacterial against the acute hepatopancreatic necrosis disease (AHPND)

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Abstract. Herbal extracts are considered as alternative approaches for sustainable aquaculture. The present study aimed to evaluate the antibacterial potential of *Piper betle* and *Ocimum basilicum* against *Vibrio harveyi*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. Samples were extracted by the maceration method, then they were examined for their antibacterial activities against *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* by means of paper disc diffusion method. Active extracts were partitioned with different polarity solvent and tested for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The highest diameter of inhibition zone of *P. betle* (dichloromethane extract) and *O. basilicum* (aqueous extract) were 19 and 16 mm, respectively. The MICs and MBCs of the dichloromethane extract of *P. betle* ranged from 0.39 to 0.78 mg mL^{-1} and from 0.39 to 6.25 mg mL^{-1}, respectively. Meanwhile, the MICs and MBCs of the aqueous extract of *O. basilicum* ranged from 1.56 to 3.12 mg mL^{-1} and from 3.12 to 12.5 mg mL^{-1}, respectively. The results indicate that the *P. betle* and *O. basilicum* extract seem promising for a sustainable management of the diseases in the aquaculture industry.
Key Words: vibriosis, secondary metabolite, leave extract, extraction, shrimp bacterial pathogen.

Introduction. The fishing industry has experienced a rapid development, especially in the field of aquaculture (Yuan et al 2019). The aquaculture production increased from 3,193,565 tons in 2007 to 16,114,991 tons in 2017 or an increase of more than 5 times (BPS 2020). This is due to the intensification of the aquaculture. However, intensive cultivation can trigger pathogenic infections that attack aquatic organisms. Among the diseases commonly found in aquaculture areas, especially in shrimps, the vibriosis is a real issue (Karthik et al 2014; Sivakumar et al 2014). Vibriosis is known to cause a very high mortality of tiger prawns and vannamei with mortality rates reaching 95% (Nunan et al 2014) and even 100% (De Schryver et al 2014). Shrimp infected by vibriosis are characterized by a slow growth, anorexia, brownish hepatopancreas and red spots on uropods, pleopods and abdomen (Mohamad et al 2019). Several bacteria are known to cause vibriosis: *Vibrio harveyi* (Haenen et al 2014), *Vibrio alginolyticus* (Novriadi 2016) and *Vibrio parahaemolyticus* (Nunan et al 2014) which are also the main cause of Acute Hepatopancreas Necrosis Disease (AHPND). Vibriosis disease can be overcome by giving antibiotics (Harlina et al 2015).

Antibiotics have long been used by farmers in eradicating diseases that infect shrimp. Various brands of commercial antibiotics are circulating in the market such as choramphenicol, oxytetracycline, kanamycin and various other types, unfortunately commercial antibiotics have a negative impact on shrimp, the environment and consumers (Cabello 2006). Even some export destination countries require that the exported products they receive do not contain commercial antibiotics (Supartono & Nr 2015). Therefore, other alternatives are needed in treating shrimp infected with vibriosis.

Betel (*Piper betle* L.), known as plant, has been used for their health benefits (Lei et al 2003; Shah et al 2016). *P. betle* leaf can be used as a natural antibacterial plant. The plant have some bioactive compounds such as betal-phenol, chavicol and other phenolic compounds. These compounds are known to have strong potentials as antifungal, antioxidant and antibacteria agent (Lubis et al 2020; Saraswati 2011). The antibacterial activity of betel leaf can inhibit the growth of *Escherichia coli*, *Klebsiella*, *Pasteurella*, *Salmonella* sp., *Staphylococcus aureus* and kill *Candida albicans* (Rasydy et al 2019; Reveny 2011). Betel leaf extract is often used in aquaculture to treat *Aeromonas hydrophila* infected goldfish (*Carrasius auratus*) (Meriyanti 2020; Mulia & Husin 2012), while *Ocimum basilicum* L. (Lamiaceae) is not only known in cooking but also has been used as a medicinal plant for antimicrobial, antioxidant, antinociceptive, antiviral and potential for use in treating cancer others (El-Ashram et al 2017; Khalil 2013; Silva et al 2016). In this study, the antibacterial properties of a herbal extract from the leafs of *P. betle* and *O. basilicum* were evaluated against shrimp pathogenic bacteria (*V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus*).

Material and Method

Sampling. *P. betle* and *O. basilicum* leaves were obtained from Tello Traditional Market, Batua, Makassar City (5°09'00.2"S 119°28'16.8"E). Freshly collected specimen of betel leave and basil were immediately transported to the laboratory. The samples were separated, washed and cleaned thoroughly with tap water and then with distilled water and air-dried in shade and ground into a fine powder at the Integrated Biology Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Muslim Indonesia.

Extraction. *P. betle* and *O. basilicum* leaves were prepared in accordance with the methods described in (Hamdillah et al 2019) with minor modifications. An amount of 500 g of powder for each sample was extracted using the maceration method, with 2.5 L methanol solvent (MeOH) for 24 h, and the process was repeated three times. After filtering through the Whatman No. 1 filter paper, the extracts was concentrated using a rotary evaporator (Heidolph, Germany) (Sachithanandam et al 2021). All the extracts thus prepared were stored for further analysis at 4°C.

Antibacterial assay. Shrimp bacterial pathogen like *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* were used to evaluate the antibacterial activity of extracts from *P. betle* and *O. basilicum*. All isolates were obtained from the Research Institute for Coastal Aquaculture, Maros. The bacteria were cultured and maintained on nutrient agar slants (Oxoid, UK). Bacterial inhibition assay used the diffusion method on double layer agar (Isnansetyo & Kamei 2003). All inoculum of 106 cells mL^{-1} based on standard McFarland 0.5 were transferred into TSB (Oxoid, UK) added with 0.7% agar. Then, the mixtures were poured into TSA (Oxoid, UK). Each sterile paper disc (6 mm diameter, Macherey Nagel, Germany) were impregnated with extracts of a concentration of 50 mg mL^{-1}, placed on the surface of inoculated agar and incubated at 30°C for 24 h. The antibacterial activity was measured based on the diameter of the inhibition zone.

Partition of crude extract. The crude extract of *P. betle* and *O. basilicum* was further partitioned using immiscible solvent: 50 g of active extract was partitioned using solvents with increasing polarity, based onn-hexane, dichloromethane and distilled water, to obtain hexane, dichloromethane and aqueous solutions, respectively. All the organic fractions of the extract (*n*-hexane, dichloromethane and aqueous extracts) were tested for their antibacterial activity as described previously and stored at 4°C prior to use.

Determination of MIC and MBC. The MIC and MBC tests were carried out on the extract with the highest antibacterial activity. Dichloromethane extract of *P. betle* L and aqueous extract of *O. basilicum* were evaluated for MIC and MBC. The MIC and MBC tests were based on a diffusion method on double layer agar (Isnansetyo & Kamei 2003; Isnansetyo & Kamei 2003) and the bacteria tested were *V. alginolyticus*, *V. harveyi*, and *V. parahaemolyticus*. Fractions were tested at different concentrations (50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 mg mL^{-1}), dripped onto a paper disc, then air-dried in an incubator at 30°C before being placed on soft agar media. The diameter of the inhibition zone formed after 24 hours was observed and measured. The lowest concentration of the active extract indicated the zone of inhibition corresponding to the MIC value. On the other hand, the extracts were incubated again at 30°C for 24 h to determine the MBC, as the lowest concentration of drug or extract, which did not produce any bacterial colonies on the agar plate.

Results. In the present study, *P. betle* and *O. basilicum* extracts against the growth of *V. harveyi, V. alginolyticus* and *V. parahaemolyticus* were evaluated. *P. betle* leaves and basil *O. basilicum* leaves were obtained from the Tello Traditional Market, Batua, Makassar City (5°09'00.2"S 119°28'16.8"E). The research on the antibacterial potential of *P. betle* and *O. basilicum* was carried out in several stages. *P. betle* and *O. basilicum* leaves were subjected to an extraction process with methanol solvent and yielding a percentage of 9.9% and 13.93% of crude extract.

Extraction of crude P. betle and O. basilicum. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone after incubating the plates at 30 °C for 24 h. The antibacterial assay of the bioactive compound of *P. betle* and *O. basilicum* extract showed an efficient suppression of the growth of bacterial disease in shrimp. Leaf extract of *P. betle* and *O. basilicum* had a remarkable antibacterial activity with maximum zones of inhibition about 14 mm against *V. harveyi*, 17 mm against *V. Alginolyticus*, and 16 mm against *V. parahaemolyticus* (Table 1).

Table 1

Antibacterial activities of leaves crude extract from *Piper betle* and *Ocimum basilicum* against different bacterial

Plant crude extract	*Diameter of inhibition zone (mm)*		
	V. harveyi	*V. alginolyticus*	*V. parahaemolyticus*
Piper betle	14	14	16
Ocimum basilicum	14	17	15

Antibacterial activity of the partitioned extract. Crude methanol extract of *P. betle* and *O. basilicum* were fractionated based on the polarity of the compound. The solvents used were *n*-hexane, dichloromethane and distilled water. The n-hexane solvent would attract non-polar compounds. Dichloromethane solvent would attract semi-polar compounds and distilled water would attract polar compounds. The antimicrobial activity of the partitioned extract is presented in Table 2. Only the dichloromethane partition extract of *P. betle* showed antibacterial activity. On the other hand, only the aqueous partition extract of *O. basilicum* showed antibacterial activity. Based on the results of the study in table 1. that dichloromethane extract of *P. betle* was able to inhibit the growth of *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* with inhibition zone diameters of 19, 16 and 15 mm, respectively. Meanwhile, aqueous extract and n-hexane of *P. betle* did not inhibit the growth of all test bacteria.

Table 2
Antibacterial activity test of extracts of n-hexane, dichloromethane and aqueous extract of *Piper betle* and *Ocimum basilicum* against bacterial pathogens

Plant samples	*Extract*	*Diameter of inhibition zone (mm)*		
		V. harveyi	*V. alginolyticus*	*V. parahaemolyticus*
Piper betle	Aqueous	-	-	-
	Dichloromethane	19	16	15
	n-Hexane	-	-	-
Ocimum basilicum	Aqueous	16	13	15
	Dichloromethane	-	-	-
	n-Hexane	-	-	-

MIC and MBC. In the current study, the MIC and MBC values were determined from the partitioned extract. The highest antibacterial activity was determined for its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Table 3). The MIC and MBC were evaluated against *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* using a diffusion method on double layer agar (Isnansetyo & Kamei 2003; Isnansetyo & Kamei 2003). Dichloromethane extract of *P. betle* and aqueous extract of *O. basilicum* were evaluated with different concentrations (50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 mg mL^{-1}).

Table 3
Minimum concentration (MIC) test results

Bacteria	*MIC (mg mL^{-1})*		*MBC (mg mL^{-1})*	
	O. basilicum (Aqueous)	*P. betle (Dichloromethane)*	*O. basilicum (Aqueous)*	*P. betle (Dichloromethane)*
V. harveyi	1.56	0.39	3.12	0.39
V. alginolyticus	3.12	0.78	12.5	6.25
V. parahaemolyticus	1.56	0.39	6.25	0.78

Based on Table 3, the MIC value of *P. betle* (0.39 mg mL^{-1}) was lower than the MIC value of *O. basilicum* (1.56 mg mL^{-1}). Likewise, MBC of *P. betle* (0.39 mg mL^{-1}) was lower than MBC of *O. basilicum* (3.12 mg mL^{-1}). The lower the concentration of the extract that can inhibit or kill bacteria, the better the extract. Besides, the MIC and MBC values of the dicholoromethane extract of *P. betle* were the same against *V. harveyi*, meaning this bacteria can be inhibited and killed at the same concentration of the plant extract.

Discussion. Shrimp aquaculture is one of biggest industry in the world. Herbal plants, like *P. betle* and *O. basilicum* are known well as natural medicines and may play a role in the development of new phytomedicine drugs to be used for the treatment of diseases. The data summarized showed a broad spectrum of antibacterial activity of *P. betle* and *O. basilicum* against *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus*. The extracts showing a diameter of inhibition zone less than 10 mm were classified as weak antimicrobial (Jang et al 2007) while the extracts with an inhibition zone of more than 15 mm were classified as strong antimicrobials (Al-Madhagi et al 2019). A strong antibacterial activity was shown by *P. betle* against *V. parahaemolyticus* and by *O. basilicum*against *V. alginolyticus* and *V. parahaemolyticus*. *P. betle* and *O. basilicum* had an antifungal and antimicrobial activity against pathogens (gram positive and negative) (Pandey et al 2014; Sarma et al 2018; Voon & Hussin 2014). The ethanolic crude extract of betel leaves is a potential alternative to antibiotics against the *V. alginolyticus* infection (Othman et al 2018). The opportunistic pathogen *Vibrio* may cause significant losses in crustacean farms (de Souza & Wan 2021). Morever, AHPND is known to be caused by strains of Vibrio parahaemolyticus that contain a plasmid of a unique virulence (Sachithanandam et al 2021). The infection occurrs because *V. parahaemolyticus* contains two toxin genes, namely PirA and PirB (Lai et al 2015). These bacteria also trigger the occurrence of white stools or White Feces Disease (WFD) in shrimp (Supono et al 2019). *V. parahaemolyticus* is generally found not only in brackish waters but also in marine waters (Mohamad et al 2019).

Most bioactive compounds isolated from *P. betle*, such as the carvacrol, eugenol, hydroxychavicol and chavibetol (an isomer of the eugenol) are highly volatile and show a poor solubility in the aqueous phase (Kurniasari et al 2021; Othman et al 2018; Zamakshshari et al 2021). They are able to suppress the growth of *Vibrio* sp. (Maharajan & Sajin 2011) and of some fungi like *Candida albicans* (Maharajan & Sajin 2011; Shah et al 2016; Zamakshshari et al 2021) and *Aspergillus niger* (Sarma et al 2018; Zamakshshari et al 2021). Meanwhile, Ahmed et al (2021) reported that *O. basilicum* inhibits the growth of nine known fish pathogens: four gram-positive bacteria (*Enterococcus faecalis*, *Bacillus* sp., *Streptococcus agalactiae* and *S. aureus*) and five Gram-negative bacteria (*Klebsiella pneumoniae*, *A. hydrophila*, and *V. alginolyticus*).

The extract of *P. betle* has phenolic compounds and derivates that can suppress the growth of Propionilbacterium acne. Phenolic substances are the most common secondary metabolites with microbial growth inhibitory abilities (Lubis et al 2020). Phenolic compounds suppress the growth of pathogenic microorganisms by inhibiting (bacteriostatic) and killing (bactericidal) the microbes or by denaturing the three-dimensional bacterial proteins (Noventi & Carolia 2016), disrupting the covalent structure of Gram-positive bacteria. It will lead to the destruction of the wall of bacteria cells. Another report explained the activity of the carboxyl group, forming complexes with extracellular and soluble proteins of bacteria, which results in the loss of their infectivity (Lubis et al 2020).

Flavonoid and tannin compounds also contribute to the antimicrobial activity of the *P. betle* leaf extract. The inhibitory effect of tannin is due to the tannic acid. The mechanism proposed was due to its ability to create a change in its potassium concentration, determining the enzymatic reactions' inhibition and the enzyme production's inhibition. The flavonoid mechanism is by disrupting the concentration of potassium in the Gram-positive bacteria which leads to the dysfunction of their cytoplasm membrane (Lubis et al 2020).

On the other hand, the antibacterial activities of *O. basilicum* were presented in Table 2, the best being oserved for the aqueous solvent extracts, which inhibited the growth of *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* with a maximum diameter of the inhibition zone of 16, 13 and 15 mm, respectively. The extract of *O. basilicum* exhibited a potent inhibitory action on *A. hydrophila, A. veronii, Pseudomonas fluorescens and Streptococcus agalactia* (Dawood et al 2021) and on shrimp pathogens, such as *Pseudomonas aeruginosa*, *S. aureus*, and some *Vibrio* pathogen (Velmurugan et al 2010).

Bioactive compounds contained in *O. basilicum* such as linalool and eugenol are responsible for the antimicrobial activity against three species of gram-positive bacteria (*B. subtilis*, *Clostridium defficile* and *S. aureus*), three species of gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumonia* and *Salmonella typhi*) and three species of fungi *(Aspergillus niger*, *A. flavus* and *Candida albicans*) (Rubab et al 2021; Silva et al 2016). Linalool (an essential oil) is a bioactive compound protecting the cells from the dangerous impact of aflatoxins through two possible mechanisms, firstly by decreasing the DNA binding formation of aflatoxins and secondly by reacting with the ROS increased by aflatoxins (Neveen et al 2017). The antibacterial and antifungal activity of the eugenol in *O. basilicum* can be ascribed to the presence of a free hydroxyl group in the molecule. The hydroxyl group of the eugenol is thought to bind to the proteins, preventing the enzymatic actions. These effect is associated with the antiinflammatory and antioxidant activities. The eugenol has also shown an excellent antifungal activity against dermatophytes, imperfect filamentous fungi and pathogenic yeasts and an efficient activity against a wide range of gram-negative and gram-positive bacteria (Marchese et al 2017; Snoussi et al 2016). In other reports, the compounds assumed as antimicrobials are the 2,6 octadiene 1,8 diol, phytol, camphor, exo methyl champenilol linalool oxide, cis geraniol, cis carveol (Solikhah et al 2016).

According to Table 3, the herbal extracts showed a different inhibition against the shrimp pathogens. The MIC value of the dichloromethane extract of *P. betle* (0.39 mg mL^{-1}) was better than in the aqueous extract of *O. basilicum* (1.56 mg mL^{-1}). Nevertheless, *O. basilicum* extract is a potential alternative as antibiotic against vibriosis. *P. betle* extract demonstrated the highest effectiveness against *V. alginolyticus* and *V. harveyi*, both with MIC values of 0.39 mg mL^{-1}, whereas against *V. alginolyticus* the MIC values were of of 0.78 mg mL^{-1}. *P. betle* can inhibit the growth of *A. hydrophila* with a MIC of 0.37 mg mL^{-1} (Ahmad et al 2021). Moreover, Nafiqoh et al (2020) reported a MIC of 2 mg mL^{-1} for the *P. betle* against *A. hydrophila*

Extracts of *P. betle* and *O. basilicum* can trigger the innate immune response (such as bactericidal activity, serum lysozyme, respiratory burst activity and hematocrit levels) and thus they can reduce the fish and shrimp mortality (El-Ashram et al 2017; Kumar et al 2013; Nafiqoh et al 2020). The right dosage of the methanol extract of *P. betle* is considered optimal for improving the growth performance, enhancing immunity levels and providing antibacterial properties against the selected bacteria in Nile tilapia fish (Mohtar et al 2021). A treatment with the *P. betle* leaf extract increases the survival rate of the catfish to 66.67 to 86.11% compared to only 30.55% for controls (Mulia & Husin 2012). The leave extract of *P. betle* has a significant effect on the survival and on the histopathology of the vaname’s hepatopancreas (Annisa 2017). Besides, in vivo analysis showed that the supplemented commercial feed for *P. betle* protects the *Penaeus vannamei* postlarvae against the *V. harveyi* infection after seven days of treatment (Guzman et al 2022) and inhibited the quorum sensing mediated bioluminescence production and biofilm formation in *V. harveyi* (Srinivasan et al 2017). In vivo assessment showed that *O. basilicum* inhibited the growth of five different shrimp pathogens (*A. hydrophila*, *S. aureus*, *P. aeruginosa*, *V. harveyi* and *V. parahaemolyticus*) and improved the survival rate in the *Penaeus monodon* shrimp post-larvae culture (PL 25) (Velmurugan et al 2010).

Conclusions. *P. betle* and *O. basilicum* leaves exhibit bacterial inhibition properties against various pathogens affecting economically important aquaculture species of shrimp. MIC and MBC values of *P. betle* leaves reached 0.39 and 0.39 mg mL^{-1}, respectively. MIC and MBC values of *O. basilicum* leaves reached 1.56 and 3.12 mg mL^{-1}, respectively. The use of *P. betle* and *O. basilicum* extracts seem promising for a sustainable disease management in the aquaculture industry.

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