

SCREENING ANTIBACTERIAL EFFECTS OF VIETNAMESE PLANT EXTRACTS AGAINST PATHOGENS CAUSED ACUTE HEPATOPANCREATIC NECROSIS DISEASE IN SHRIMPS

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ABSTRACT

Objectives: The objectives are aimed to investigate the antibacterial properties of five Vietnamese medicinal plants against acute hepatopancreatic necrosis disease (AHPND)-caused bacterial pathogens, to verify their potentials to apply as a new treatment therapy.

Methods: Extracts from plants, such as *Psidium guajava* leaf, *Piper betle* L. leaf, *Phyllanthus amarus* leaf, *Rhodomyrtus tomentosa* seed, and *Allium sativum* bulb, were tested against three AHPND-caused bacteria. Agar infusion and broth dilution methods were employed to evaluate extract *in vitro* antibacterial effects, while experiments with cultured whiteleg shrimps were applied to access their safety when applied *in vivo*. High-performance liquid chromatography (HPLC) analysis was applied to identify components in the extracts.

Results: *P. amarus* and *R. tomentosa* extracts exerted the strongest inhibition on tested bacteria. Other extracts, including *P. betel* and *P. guajava*, were less effective, while *A. sativum* showed no effects against bacteria. In safety assessment experiments, we observed that only crude extracts of *R. tomentosa* and *A. sativum* were safe, while others significantly reduced their survival rates. HPLC showed that extracts of high antibacterial properties had rich phenol constituents. In addition, the phenolic profile of *R. tomentosa* showed the presence of piceatannol.

Conclusion: Considering both of antibacterial effects and safety properties altogether, we concluded that among the five examined plant materials of this study, *R. tomentosa* had the highest potential to apply in AHPND treatment, as only this plant showed the high effects on pathogenic bacteria while were still safe for host aquatic shrimps.

Keywords: Medicinal plant, *Rhodomyrtus tomentosa*, Antibacterial effect, Acute hepatopancreatic necrosis disease, Acute hepatopancreatic necrosis disease, Shrimp.

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INTRODUCTION

A new lethal disease, termed early mortality syndrome or acute hepatopancreatic necrosis disease (AHPND), has been emerged in Vietnam and caused a severe damage to the country shrimp aquaculture [1,2]. After the outbreak of China in 2009, this disease spreads sequentially to Vietnam (2010), Malaysia (2011), Thailand (2012), and Mexico (2013) [3]. AHPND causes mortality of as high as 100% [4] and has destroyed up to about 80% of the shrimp products in some affected areas [5]. In 2013, the causative agent of AHPND was identified as unique isolates of *Vibrio parahaemolyticus* [1]. In addition, researchers have recently found that virulence genes on plasmid might be transferred not only among *V. parahaemolyticus* strains but also to different bacterial species. These bacteria were identified as *Vibrio harveyi*, isolated from affected shrimps in Vietnam and labeled as *Vibrio harveyi* KC13.17.5 (*V. harveyi* KC13.17.5) [4]. Therefore, pathogenic bacteria of AHPND outbreaks in Vietnam have been so far identified as not only *V. parahaemolyticus* but also *V. harveyi* KC13.17.5. Based on this background, our study decided to test plant effects on AHPND pathogenic bacteria by examining their effects with *V. parahaemolyticus* KC12.020, *V. parahaemolyticus* KC13.14.2, and *V. harveyi* KC13.17.5, which had been isolated from AHPND-affected shrimps in Vietnam [4,6].

As microbial disease in aquaculture industries makes serious financial loss, and the antibiotic application in treatment shows many side effects; the search for an alternative method, such as medicinal plants, has been

proposed to improve the quality and sustainability of aquaculture production [7-9]. Researchers have observed that medicinal plants not only exert high antimicrobial properties on aquatic bacteria but also have positive effects on the growth and survival rates of aquatic animals, and therefore, proposed their use as a high potential therapy to replace chemotherapeutic molecule use for aquaculture [7,10]. In case of the new emerging AHPND of shrimps, several remedies to control the disease have been proposed, but a definite solution remains unclear [11]. In addition, antibiotic resistance has been detected on AHPND pathogenic bacterial strains, including those were isolated from the affected shrimps in Vietnam [11], suggesting that investigation on other treatment methods, such as the use of medicinal plants, would be useful in the search for an effective solution.

In this present study, we tested antibacterial effects of five Vietnamese medicinal plants, such as *Psidium guajava* leaf, *Piper betle* L. leaf, *Phyllanthus amarus* leaf, *Rhodomyrtus tomentosa* seed, and *Allium sativum* bulb, against the three pathogenic AHPND bacterial strains isolated in Vietnam, to evaluate their therapeutic potentials. In addition, we also characterized the compounds in plants and performed feeding experiments with shrimps to assess their *in vivo* safety. In our study, we decided to choose the five plants because Vietnamese ethnic medicine had described them as therapies for diseases associated by bacterial infections [12, 13], and they had shown high effects on other aquatic bacteria in our previous results [14-16].

METHODS

Source of AHPND-caused bacterial pathogens

Virulent bacterial strains such as *V. parahaemolyticus* KC12.020, *V. parahaemolyticus* KC13.14.2, and *V. harveyi* KC13.17.5 used in this study were isolated from AHPND-affected shrimps during this disease outbreak in Vietnam. Their biochemical, virulent, and molecular analysis had been previously performed [4,17]. After being glycerol stocked, these bacteria were preserved at -80°C and kept in our laboratory, Department of Aquatic Animal Diseases, Research Institute for Aquaculture No. I, Vietnam.

Confirmation of AHPND-caused bacteria by polymerase chain reaction (PCR)

To confirm AHPND-caused bacterial strains at genomic level, DNAs were extracted from the bacterial strains which grown on nutrient broth growth medium and used as DNA templates for the PCR analysis employing AP3 primers (Forward: 5'Forward: rs NA templates for ow and reverse: 5' versed: rs NA templates forand toxin gene primers (forward: 5rw GTGGAAATGGTGAACCTTGC-3' and reverse: 5'- TACGAGCATTGTTAGGGGTTA-3'), provided from laboratory of Genome Science, Tokyo University of Marine Science and Technology. PCR conditions were performed for 30 cycles and as follows: An initial denaturation at 94°C for 5 min and each cycle with denaturation at 94°C for 30 s, annealing at 53°C for 30 s, extension at 72°C for 40 s, and final extension at 72°C for 5 m. PCR amplified products were resolved in a 1% agarose gel containing ethidium bromide by electrophoresis and visualized under UV light. The positive results showed a band with a size of 336 bp for AP3 primers [18] and a size of 630 bp for toxin primers.

Plant materials and extraction

Five plants, including *P. guajava*, *P. betle*, *P. amarus*, *R. tomentosa*, and *A. sativum*, were collected from Vietnam. Their identities were confirmed by Dr. Tho Thi Bui based on voucher specimens that had been deposited at Vuon Duoc Lieu Thu Y Herbarium, Vietnam National University of Agriculture in Vietnam. After collecting, the plant materials were washed, preliminarily shade-dried before further dried at 50°C for 15 h. They were then ground individually into powders with a particle size <0.1 mm, put in airtight plastic bags kept in dried cool chambers. Samples were tested within 6 months from the preparation time. *P. guajava*, *P. betle*, *P. amarus*, and *A. sativum* were extracted by 70% ethanol with 1/10 ratio of plant powder/ethanol. Followed Lai *et al.* [19] recommendation, *R. tomentosa* was extracted by ethanol 79% with 1/20 ratio of plant powder/ethanol. Solutions were centrifuged at 6,000 rpm for 10 m at 4°C . The supernatants were concentrated by a rotatory evaporator at 40°C , and extracts were dried with a lyophilizer before being stored at 4°C until use [19].

Preparation of bacterial concentration

Three bacterial strains, *V. parahaemolyticus* KC12.020, *V. parahaemolyticus* KC13.14.2, and *V. harveyi* KC13.17.5, were taken from glycerol stocks at -80°C and streaked on the thiosulfate citrate bile salt plates and incubated at 29°C for 24 h. After that, a single colony of each strain was selected and cultured individually in 50 ml nutrient broth supplemented with 2% of NaCl and incubated at 29°C for 15 h in a shaking incubator (shaking speed: 200 rpm). The concentrations of bacteria were calculated based on optical density (OD) values obtained with a spectrophotometer at the wavelength of $\lambda = 600$ nm. The bacterial concentrations were adjusted to 10^8 CFU/ml in the test with plant extracts.

Investigation of antibacterial effects against AHPND-caused vibrio bacterial strains

Evaluation of antibacterial effects with agar disc diffusion method

Antibacterial effects were determined through measuring the inhibitory zones induced by the extracts. In brief, plant extracts were dissolved in dimethyl sulfoxide (DMSO) to make concentrations of 10 $\mu\text{g}/\mu\text{l}$,

15 $\mu\text{g}/\mu\text{l}$, 20 $\mu\text{g}/\mu\text{l}$, 25 $\mu\text{g}/\mu\text{l}$, and 30 $\mu\text{g}/\mu\text{l}$ and examined with bacteria, followed the agar disc diffusion method of Bauer *et al.* [20]. The extract disc contained 100 μl of extract, while the positive control disc contained doxycycline (30 μg) or ampicillin (10 μg) and the negative control disc contained only 100 μl DMSO. The discs were placed on Mueller Hinton agar plates and incubated at 29°C for 24 h. Diameters of inhibitory zones induced by the extracts were interpreted as follows: Resistant (12 mm or less), intermediate (13–15 mm), and susceptible (16 mm or more), followed the standard that has been used to evaluate effects of plants on bacteria [14].

Evaluation of antibacterial effects by broth dilution method

Antibacterial effects were determined through the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the plant extracts. In brief, extracts were initially dissolved in DMSO to make the stock solutions and then diluted into the nutrient broth to make the two-fold diluted concentrations that ranged from 19 $\mu\text{g}/\text{ml}$ to 5000 $\mu\text{g}/\text{ml}$ to test with bacteria. MIC values were determined as the lowest concentration that completely inhibited the growth of bacteria after 24 h of incubation at 29°C , and MBC values were determined as the lowest concentrations that inhibited the bacterial growth after they were plated again on nutrient agar and further incubated for 24 h.

High-performance liquid chromatography (HPLC) analysis of plant extracts

The method used for HPLC of extracts was modified from Lai *et al.* [21]. After being filtered through 0.45 μm pore-size syringe (Phenex™-NY, Utrecht, The Netherlands), 20 μL of extract was analyzed using a 150 \times 4.6 mm i.d.; Kinetex 5 μm C18 column equipped with a guard column of the same type (Phenomenex, Netherlands). The mobile phases were: (A) H_2O with 0.1% formic acid, and (B) acetonitrile with 0.1% formic acid. The gradient conditions were as follows: 0–5 min, 0–15% B; 5–20 min, 15–20% B; 20–30 min, 20–100% B; 30–35 min, 100% B; 35–40 min, 100–0% B; and 40–42 min, 0% B. Other chromatographic conditions were as follows: Flow rate: 0.3 ml/min, column temperature: 30°C , and run time: 42 min. The identification of compounds in extracts was followed by the study of Robards *et al.* [22].

Safety assessment of plant extracts

Preparation of whiteleg shrimp

Whiteleg shrimp (weigh of 2–3 g) bought from a shrimp farm in Nghe An Province was stocked in a seawater aquaria to assess their disease-free health status for 7 days. We divided the healthy shrimps into six experimental groups ($n=90$ per group, except for the control group in which $n=30$), and each group housed in three subgroups of nine glass tanks ($n=10$ per tank and 3 tanks for sub-group) containing 50 L of artificial seawater and equipped with an air supply system. Each subgroup was nominated for each tested concentration of plant extract, and three tanks for subgroup indicated the triplication. The shrimps were maintained under the following conditions: $26\text{--}28^{\circ}\text{C}$, $\text{pH} = 7.5\text{--}8.5$, $\text{DO} \geq 4$ mg/L, and 25 ppt.

Preparation of feed pellets coated with plant extracts

Five tested plant powders were separately mixed with distilled water, sprayed onto shrimp feed pellets, and mixed before coating with cod liver oil (Merck). Mixing ratio of plant extract powders and pellets was optimized so that concentrations of each plant extracts were 50 mg/g pellet, 75 mg/g pellet, and 100 mg/g pellet. The ratio of cod liver oil to pellets was 2 ml/100 g. Those feed pellets were stocked for maximum 15 d at 4°C until use.

Safety assessment of plant extracts on whiteleg shrimps

Pellets without or with plant extracts were used to feed shrimps at the amounts equivalent to 1–2% of shrimp body weights. For each plant material, nine tanks of shrimps were used for extract-coated pellets at three different concentrations, and three tanks were used for control.

All experiments were performed in triplication, and survival rates were recorded every day, in the period of 14 days.

Statistical analysis

Data were expressed as mean ± standard error (mean ± SEM). One-way ANOVA followed by *post hoc* Bonferroni test was used to compare the inhibitory zones induced by different extract concentrations and the survival rates of different groups in safety assessment test. Two-factor ANOVA followed by *post hoc* Bonferroni test was used to compare the inhibitory zones induced by different extracts. In all analysis, significance was established when probability level was equal to or <5% (p<0.05).

RESULTS

PCR analysis of AHPND-caused bacterial strains

Applying AP3 detection method and toxin primer for AHPND using PCR analysis, we confirmed that three strains, including *V. parahaemolyticus* KC12.020, *V. parahaemolyticus* KC13.14.2, and *V. parahaemolyticus* sp. KC13.17.5, are the pathogenic bacteria that cause AHPND in shrimps (Fig. 1a and b). PCR results of those bacteria showed bands that were the same as that of the positive control DNA at 336 bp when using AP3 primers (Fig. 1a) and at 630 bp when using toxin primer (Fig. 1b).

Antibacterial effects of plant extracts against AHPND-caused Vibrio bacterial strains

Inhibitory zones (mm of diameter) induced by five plant extracts against three AHPND-caused bacterial strains are shown in Table 1. We observed that while *P. guajava*, *P. betle*, *P. amarus*, and *R. tomentosa* exerted inhibitory effects at various levels, depending on bacterial strains and concentrations, while *A. sativum* showed no effects, as it induced no inhibitory zones at all tested concentrations. In addition, the inhibition of *P. guajava*, *P. betle*, *P. amarus*, and *R. tomentosa* was dose-dependent, because following the increment in applied concentrations, there were also significantly increments in their induced inhibitory zones (Table 1). Among 5 tested plants, the effects of *P. amarus* and *R. tomentosa* were remarkable, because their extracts always induced the inhibitory zones that represented bacterial susceptibility (>16 mm) for all 3 tested strains, while others, such as *P. guajava* and *P. betle*, induced such kind of inhibitory zones for only one or two bacteria, respectively. The results were further evident with Fig. 2, which compares the inhibitory zones induced by the four extracts and shows that regardless of tested concentrations, *P. amarus* and *R. tomentosa* always represented the highest antibacterial effects, as shown by their significantly larger inhibitory zones (Fig. 2). The determination of MIC and MBC by a broth dilution method, as shown in Table 1, also further confirmed the high effects of *P. amarus* and *R. tomentosa*, as regardless of bacterial strains, the two extracts' MIC and MBC values were always lower than those of others, such as *P. guajava* and *P. betle* (Table 1). The results thus demonstrated their stronger effects in both of inhibitory and bactericidal activities (Table 2).

Chromatographic, spectral characterization and identification of main compounds in potential plant extracts

The summary of peaks in chromat profiles of extracts from five plants is shown in Table 3. According to Table 3, major peaks in profiles of four extracts of high antibacterial effects, including *P. guajava*, *P. amarus*, *P. betle*, and *R. tomentosa* (As shown in Table 1. Inhibitory zone (mm) induced by plant extracts against AHPND-caused bacterial strains) were mainly identified as phenolic compounds. Based on previous results on phenolic constituents of *R. tomentosa* [19,21], peaks of *R. tomentosa* extract profile in our study were further identified as hydrolysable tannins (including Di-HHDP-galloyl-glucose, HHDP-galloyl-glucose, HHDP-digalloyl-glucose, furosin, and HHDP-trigalloyl-glucose) and stilbenes (including astringin, piceatannol, and resveratrol). In chromat profile of *A. sativum*, we observed that there were two minor peaks represented phenol compounds, while all other peaks, including the major ones, showed the retention times that are different from phenols and remained unknown in this study, due to the lack of our laboratory authentic standards (Table 3).

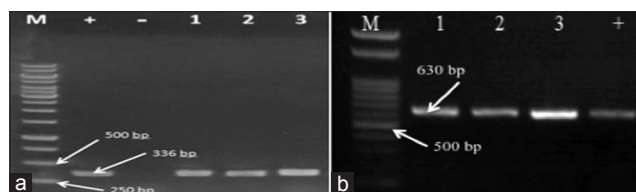


Fig. 1: Confirmation of acute hepatopancreatic necrosis disease-caused bacterial strains by PCR analysis (a) using AP3 primer and (b) using toxin primer. (1) *Vibrio parahaemolyticus* KC12.020, (2) *V. parahaemolyticus* KC13.14.2, (3) *V. parahaemolyticus* sp. KC13.17.5, (4) distilled water, (+) positive (control) DNA template and (M) marker 100 bp

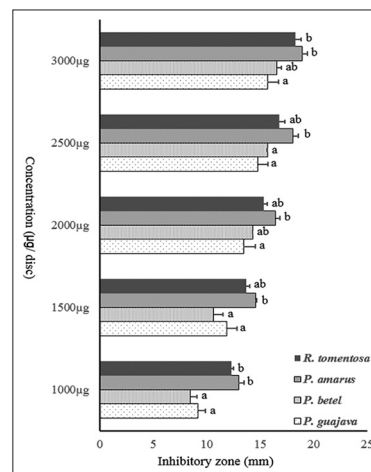


Fig. 2: Means of inhibitory zones of extracts from different plant materials to three acute hepatopancreatic necrosis disease-caused bacterial strains. Each value was mean of inhibitory zones of each extract at each concentration of three bacteria. Values with different superscripts in each concentration are significantly different (p<0.05) by two-factor ANOVA followed by Bonferroni post hoc analysis

Safety assessment of plant extracts

The safety assessment results of plant extracts on cultured whiteleg shrimps are shown in Fig. 3. The survival rates of shrimps fed with *R. tomentosa* and *A. sativum* at concentrations of 50 mg/g and 75 mg/g remained 100% in all experimental period (14 d), while those of shrimp fed with *P. guajava*, *P. amarus*, and *P. betle* were significantly decreased in all tested concentrations. These results indicated that only extracts from *R. tomentosa* and *A. sativum*, at the concentrations of <100 mg/g, were safe to be directly applied in the shrimp feeding.

DISCUSSION

There has been a number of studies reported antibacterial effects of medicinal plants on *V. parahaemolyticus* and *V. harveyi* [7,23-29], but our study represents the second attempt to investigate plant effects on AHPND pathogenic bacteria, followed one report in 2014 [30]. In addition, it is the first time that plants were tested against the AHPND pathogenic bacterium that was different from *V. parahaemolyticus* and was closest to *V. harveyi* and the *V. harveyi* KC13.17.5 strain. We observed that four of five examined plants, including *P. guajava*, *P. amarus*, *P. betle*, and *R. tomentosa*, showed antibacterial activities at different levels. These results were similar to previous studies that reported these plants' effects on bacteria [31-37] and further give evidence to explain their Vietnamese ethnic applications in bacterial diseases [12,13]. In addition, our results demonstrated that among all tested plants, *P. amarus* and *R. tomentosa* had outstanding effects on AHPND-caused bacteria, as they always represented the strongest activities, regardless of applied investigation methods (agar disc diffusion or broth dilution) and also regardless of tested strains (*V. parahaemolyticus*

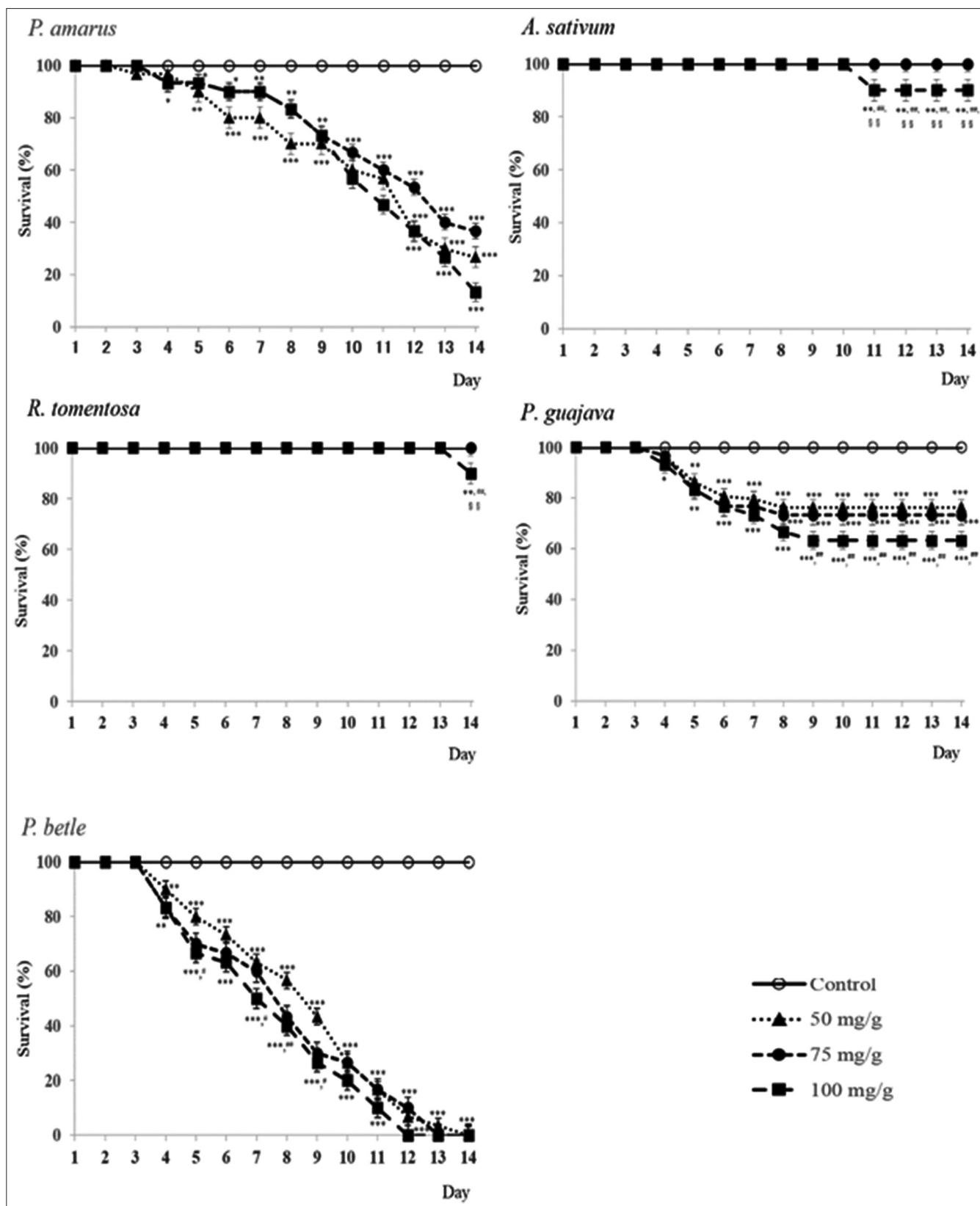


Fig. 3: Survival rates of cultured whiteleg shrimps fed without extract (○) and with extract at different concentrations (▲ (75 mg/g extract), ● (75 mg/g pellet), and ■ (100 mg/g pellet)). Each point represents the mean ± SEM for three experiments. The values of different groups were compared by one-way ANOVA followed with Bonferroni post hoc analysis (*p<0.05 vs. control, **p<0.01 vs. control, ***p<0.001 vs. control, #p<0.05 vs. 50 mg/g pellet, ##p<0.01 vs. 50 mg/g pellet, \$p<0.05 vs. 75 mg/g pellet, and \$\$p<0.01 vs. 75 mg/g pellet) by two-factor ANOVA followed by Bonferroni post hoc analysis

Table 1: Inhibitory zone (mm) induced by plant extracts against AHPND-caused bacterial strains

Plant extract	Concentration (µg/disc)	Bacterial strains		
		<i>V. parahaemolyticus</i> KC12.020	<i>V. parahaemolyticus</i> KC13.14.2	<i>V. harveyi</i> KC13.17.5
<i>P. guajava</i>	1000	10.3 ^a ±1.2	9.3 ^a ±3.2	8.0 ^{ab} ±1.7
	1500	13.7 ^b ±0.6	11.3 ^{bc} ±3.1	10.7 ^b ±0.6
	2000	15.7 ^{cd} ±0.6	12.3 ^{cd} ±2.3	12.3 ^{cd} ±0.6
	2500	16.7 ^{de} ±1.5	14.0 ^{de} ±1.7	13.7 ^{de} ±1.2
	3000	17.7 ^e ±0.6	14.7 ^e ±2.5	14.7 ^e ±1.5
<i>P. betel</i> L.	1000	9.7 ^a ±0.6	7.7 ^a ±1.5	8.0 ^{ab} ±1.0
	1500	11.7 ^b ±0.6	11.3 ^b ±1.5	9.0 ^b ±1.0
	2000	14.3 ^{cd} ±1.2	14.3 ^{cd} ±1.2	14.3 ^{cd} ±1.2
	2500	15.7 ^{de} ±0.6	15.7 ^{de} ±1.2	15.7 ^d ±1.2
	3000	15.7 ^e ±0.6	17.0 ^e ±1.0	17.0 ^e ±1.0
<i>P. amarus</i>	1000	12.0 ^{ab} ±1.0	13.3 ^{ab} ±1.5	13.7 ^{ab} ±0.6
	1500	14.3 ^b ±0.6	14.7 ^b ±1.2	14.7 ^b ±0.6
	2000	16.0 ^c ±1.0	17.3 ^{cd} ±0.6	16.0 ^{cd} ±1.0
	2500	18.0 ^{de} ±2.0	19.0 ^{de} ±1.0	17.3 ^d ±2.1
	3000	18.0 ^e ±1.0	19.7 ^e ±0.6	19.0 ^e ±1.7
<i>R. tomentosa</i>	1000	12.7 ^{ab} ±1.5	12.3 ^{ab} ±1.5	12.0 ^a ±2.0
	1500	13.0 ^b ±2.0	13.7 ^{bc} ±1.5	14.3 ^{bc} ±1.2
	2000	15.7 ^c ±1.2	14.7 ^{cd} ±0.6	15.7 ^{cd} ±2.3
	2500	17.3 ^{de} ±0.6	15.7 ^d ±1.5	17.3 ^d ±1.2
	3000	18.0 ^e ±0.3	17.7 ^e ±0.6	19.3 ^e ±0.6
<i>A. sativum</i>	1000	0	0	0
	1500	0	0	0
	2000	0	0	0
	2500	0	0	0
	3000	0	0	0
DMSO		0	0	0
Am (10 µg)		0	0	0
Dox (30 µg)		23.0±2.2	22.9±1.8	23.3±2.4

Amp: Ampicillin, Dox: Doxycycline. Each value represents the mean±SEM for three experiments. Numbers with different superscripts (a-e) are significantly different by one-way ANOVA followed with Bonferroni test (p<0.05). Bold letters indicate the zone diameters that are interpreted as susceptibility (≥16 mm) [14]. *P. guajava*: *Psidium guajava*, *P. betel*: *Piper betel*, *P. amarus*: *Phyllanthus amarus*, *R. tomentosa*: *Rhodomyrtus tomentosa*, *A. sativum*: *Allium sativum*, DMSO: Dimethyl sulfoxide, AHPND: Acute hepatopancreatic necrosis disease, *V. parahaemolyticus*: *Vibrio parahaemolyticus*

Table 2: MIC and MBC of plant extracts on AHPND-caused bacterial strains

Plant extract	Bacterial strains		
	<i>V. parahaemolyticus</i> KC12.020	<i>V. parahaemolyticus</i> KC13.14.2	<i>V. harveyi</i> KC13.17.5
MIC			
<i>P. guajava</i>	625	625	625
<i>P. betel</i> L.	625	625	625
<i>P. amarus</i>	312	312	312
<i>R. tomentosa</i>	312	312	156
MBC			
<i>P. guajava</i>	1250	1250	1250
<i>P. betel</i> L.	1250	1250	1250
<i>P. amarus</i>	625	625	625
<i>R. tomentosa</i>	625	625	312

MIC: Minimum inhibitory concentration, MBC: Minimum bacterial concentration, AHPND: Acute hepatopancreatic necrosis disease, *V. parahaemolyticus*: *Vibrio parahaemolyticus*, *P. guajava*: *Psidium guajava*, *P. betel*: *Piper betel*, *P. amarus*: *Phyllanthus amarus*, *R. tomentosa*: *Rhodomyrtus tomentosa*, *A. sativum*: *Allium sativum*

KC12.020, *V. parahaemolyticus* KC13.14.2, or *V. harveyi* KC13.17.5). The *in vivo* experiments on cultured shrimps showed that only two extracts, including *R. tomentosa* and *A. sativum*, were safe when applying in feeding, while others significantly affected the survival rates. By combining these safety assessment results and those of antibacterial effects, we concluded that *R. tomentosa* had the highest therapeutic potentials for the treatment of shrimp AHPND, as only this plant yielded the high effects on pathogenic bacteria but was still relatively safe for host aquatic animals. HPLC analysis showed the significant constituents of polyphenols in extracts that had antibacterial activities, suggesting that phenols might be responsible, at least in part, for their effects with bacteria. Detailed identification of phenol constituents in *R. tomentosa* showed the presence of compounds that had been known to have antibacterial properties, such as hydrolyzable tannins [38], resveratrol [39], and piceatannol [40-42]. In addition, because piceatannol is the main phenolic compound of *R. tomentosa* [21]

and its strong effects have been well established on bacteria, including the pathogenic strains of aquaculture [40-42], it is likely to speculate the significant role of this compound in *R. tomentosa* strong effects with AHPND-caused bacteria observed in the current study. However, we have not yet isolated and examined piceatannol from *R. tomentosa* on these bacteria, and the roles and mechanisms of piceatannol are still remained to identify in future researches.

Even *R. tomentosa* has a long history of application in ethnic medicine [12,13,19,43], its biological and pharmacological properties have not yet been well established, and it is listed as one of 240 "Neglected and Underutilised Crop Sciences" of Vietnam, China, Thailand, and Cambodia by the scientific project "Agrofolio" [44]. The current study highlights *R. tomentosa* antibacterial effects and safety, recommends it as a promissory candidate to treat AHPND of shrimps,

Table 3: The identification of compounds in plant extracts by HPLC analysis

Plant extract	Retention time (min)	Absorbance maxima (nm)	Assigned group/compound	
<i>R. tomentosa</i>	8.55	272	DiHHDp-galloyl-glucose	
	8.78	270	HHDp-galloyl-glucose	
	9.08	275	HHDp-digalloyl-glucose	
	9.61	275	Furosin	
	9.91	301	Astringin	
	11.31	277	HHDp-trigalloyl-glucose	
	13.86	324	Piceatannol	
	20.18	305	Resveratrol	
	<i>P. guajava</i>	8.29	274	Phenolic acid, flavanol, or tannin
		8.50	270	Phenolic acid, flavanol, or tannin
8.76		267	Phenolic acid, flavanol, or tannin	
9.24		273	Phenolic acid, flavanol, or tannin	
12.47		253, 363	Flavonol	
12.97		256, 354	Flavonol	
13.36		263, 354	Flavonol	
14.98		255, 354	Flavonol	
15.78		255, 361	Flavonol	
<i>P. betle</i> L		20.53	281	Phenolic acid, flavanol, or tannin
	27.44	279	Phenolic acid, flavanol, or tannin	
<i>P. amarus</i>	9.58	273	Phenolic acid, flavanol, or tannin	
<i>A. sativum</i>	1.63	255	Unknown	
	2.25	273	Phenolic acid, flavanol, or tannin	
	3.30	247	Unknown	
	5.00	266, 279, 286	Unknown	
	12.40	244	Unknown	
	14.10	277	Unknown	

V. parahaemolyticus: *Vibrio parahaemolyticus*, *P. guajava*: *Psidium guajava*, *P. betle*: *Piper betel*, *P. amarus*: *Phyllanthus amarus*, *R. tomentosa*: *Rhodomyrtus tomentosa*, *A. sativum*: *Allium sativum*, HPLC: High-performance liquid chromatography

and thus suggests that more research attentions would be justified to verify the therapeutic potential of this plant. In addition, further studies investigating the effects of *R. tomentosa* on shrimps in AHPND pathological conditions are still necessary to access the plant *in vivo* treatment properties, and therefore, they will be performed by the followed up research of our current project (NAFOSTED, number 106-NN.05-2013.48).

CONCLUSION

This study investigated the effects of five medicinal plant extracts on AHPND pathogenic bacterial strains, to evaluate their treatment potentials. The results showed that *P. amarus* and *R. tomentosa* had the strongest antibacterial activities, followed by *P. guajava* and *P. betle*, while *A. sativum* had no effects. On the other hand, safety assessment experiments showed that only *R. tomentosa* and *A. sativum* were safe in the feeding application. By considering the two results altogether, our study highlighted *R. tomentosa* as the medicinal plant of the highest therapeutic potential for AHPND in shrimps, because it had strong effects on pathogenic bacteria while was safe for the host animals, and therefore, this plant will be further verified in the follow-up researches of our project.

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COMPETING INTERESTS

All authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

The work presented here was carried out in the collaboration between all authors. The two first authors, Lua Thi Dang and Hai Thanh Nguyen,

were the main investigators of the study. Other authors, including Hanh Thi Nguyen, Hai Ha Hoang, Ha Thi Ngoc Lai, and Ha Thi Thanh Nguyen participated in the collection, preparation, and extraction of medicinal plants and assisted the first two authors in the HPLC analysis and *in vivo* experiments. Lua Thi Dang revised the final form of this manuscript and is the corresponding author.

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