Effectiveness of *Piper betle* and *Cymbopogon citratus*, against *Vibrio parahaemolyticus;* Pathogen Caused Acute Hepatopancreatic Necrosis Disease (AHPND) on Whiteleg Shrimp, *Penaeus vannamei*

KUA BENG CHU[1*], IFTIKHAR AHMAD ABDUL RAFI[2], NIK HAIHA NIK YUSOFF[3], FADZILAH YUSOF[4] AND IRENCE JOHN[4]

[1]National Fish Health Research Center (NaFisH), Fisheries Research Institute (FRI), Batu Maung 11960 Batu Maung, Penang, Malaysia
[2]Freshwater Production and Research Center, FRI Glami Lemi, 71650 Titi, Jelebu, Negeri Sembilan, Malaysia
[3]Marine Fish Production and Research Center, FRI Tg. Demong 22200 Besut, Terengganu, Malaysia
[4]Brackishwater Culture Research Center, FRI Gelang Patah 81550 Johor, Malaysia

**Correspondence author: kuaben01@dof.gov.my*

Abstract: Acute Hepatopancreatic Necrosis Disease (AHPND) is a bacterial disease caused by *Vibrio parahaemolyticus* and has caused mortality from 40 to 100% of farmed marine shrimp. This study focused on shrimp, *Penaeus vannamei* survival fed with herbal diets before challenged with AHPND-causing bacteria *V. parahaemolyticus*. Screening antibacterial effects of betel leaves (*Piper betle)* and lemongrass (*Cymbopogon citratus*) against pathogen *V. parahaemolyticus* showed betel leaf having moderate positive antimicrobial activity while lemongrass presented weaker antimicrobial activity. Three groups of specific pathogen free shrimps, namely group A (normal pellet), B (normal pellet mixed with betel leaves) and group C (normal pellet mixed with lemongrass) were fed for 14 days prior to challenge test with *V. parahaemolyticus* suspension of bacterial density 1 x 10^8cells/ml. Results showed a higher survival rate, 87.5% in shrimp receiving betel leaves extract compared to 37.5% in group received lemongrass. A negative and positive control group had survival rates of 100% and 50%, respectively. After 24 hours of observation, all groups except the negative control had positive AHPND lesions and bacterial *V. parahaemolyticus*. This study shows that betel leaf extract has good potential in the treatment of bacterial diseases in shrimp and that feeding shrimp with betel leaves extract has promising results for minimizing the occurrence of AHPND. Further research is needed to examine the effects of betel leaves extract at the farm level.

Keywords: AHPND/EMS, herbs extraction, challenge test, bacteria, white shrimp

***Abstrak*:** Penyakit Nekrosis Hepatopankreatik Akut (AHPND) ialah penyakit bakteria yang disebabkan oleh *Vibrio parahaemolyticus* dan telah menyebabkan kematian daripada 40 hingga 100% pada udang marin yang diternak. Kajian ini tertumpu kepada kemandirian udang, *Penaeus vannamei* yang diberi makan dengan diet herba sebelum dicabar dengan bakteria penyebab AHPND-*V. paraemolyticus*. Saringan kesan antibakteria daun sirih (*Piper betle*) dan serai (*Cymbopogon citratus*) terhadap patogen *V. parahaemolyticus* menunjukkan daun sirih mempunyai aktiviti antimikrob positif sederhana manakala aktiviti antimikrobial yang lebih lemah untuk serai. Tiga kumpulan udang bebas patogen spesifik, iaitu kumpulan A (pellet biasa), B (pellet biasa dicampur dengan daun sirih) dan kumpulan C (pellet biasa dicampur dengan serai) diberi makan selama 14 hari sebelum ujian cabaran dengan suspensi 1 x 10^8sel/ml bakteria *V. parahaemolyticus.* Keputusan menunjukkan kadar kemandirian yang lebih tinggi, 87.5% dalam udang yang menerima ekstrak daun sirih berbanding 37.5% dalam

kumpulan menerima serai. Kumpulan kawalan negatif dan positif masing-masing mempunyai kadar kemandirian 100% dan 50%. Selepas 24 jam pemerhatian, semua kumpulan kecuali kawalan negatif mempunyai lesi AHPND positif dan bakteria *V. parahaemolyticus*. Kajian ini menunjukkan bahawa ekstrak daun sirih mempunyai potensi yang baik dalam merawat penyakit bakteria pada udang dan pemberian makanan dengan ekstrak daun sirih berpotensi untuk meminimumkan kejadian AHPND. Kajian lanjut diperlukan untuk mengkaji kesan ekstrak daun sirih di peringkat ladang.

Introduction

Acute Hepatopancreatic Necrosis Disease (AHPND) of shrimp is a type of disease caused by a strain of *Vibrio parahaemolyticus* that is bound to release potent toxins PirAvp/PirBvp (Han et al., 2015). Accordingly, the toxins cause tissue loss and hepatopancreatic dysfunction, which result in mortality (Zorriehzahra and Banaederakhshan, 2015). AHPND often happens 35 days or less after postlarvae (PL) are stocked in ponds. Extremely high mortality rates were observed in infected shrimp ponds in their early stage of growth cycles. AHPND was reported in China, Vietnam, Malaysia, Thailand, Mexico, and the Philippine in 2009, 2010, 2011, 2012, 2013 and 2014 respectively (Tran et al., 2013; Joshi et al., 2014; Soto-Rodriguez et al., 2015; Dabu et al., 2015; Kua et al., 2016). Losses due to EMS/AHNPD was reported in Peninsular Malaysia, with estimation losses based on production at RM1.6 billion (USD 0.49 billion) between 2011 to 2013 (Kua et al., 2018).

Since the first occurrence of AHPND in 2009, there were numerous remedies solution have been put in practice by local shrimp farmers. Among them were replacing the culture species to fish, introducing multitrophic species, using garlic juice, and putting charcoal in the pond. In order to minimize losses due to disease outbreaks or mortality, shrimp farmers used various chemicals, including antibiotics. Use of antibiotics is permitted in Malaysia when prescribed by a competent authority and controlled under specific policies related legislations covering most of the antimicrobial used pathways (MOH, 2017). As well as included the management and control of antimicrobial in aquaculture under the National Fish Health Strategy 2018-2022 (DOF, 2018). The Poison Act, Feed Act, Fisheries Act, and Food Act are the primary acts that address AMR and AMU management in Malaysia. According to Defoirdt et al., (2011), antibiotics had been applied to shrimp farm production as a bacterial disease treatment. Unfortunately, prolonged and irresponsible usage of antibiotics in the shrimp industry can contribute to bacterial resistance and is not cost-efficient in the long term. Han et al., (2015) highlighted on antibiotic resistance detected on AHPND pathogenic bacterial strains in Vietnam. Due to this condition, an alternative approach, such as natural substance from herbs for the prevention of bacterial disease, should be emphasized.

Furthermore, the growing concern about the usage of antibiotics toward human food safety has led to the development of control foodborne pathogens by using antimicrobial compounds. Alzoreky et al., (2002) reported that antimicrobial compounds in herbs were found to possess antimicrobial activity. Natural substance from betel leaves (*Piper betle*) and lemongrass (*Cymbopogon citratus*) extracts are known to have antimicrobial activity against pathogenic bacteria in human (Liao et al., 1999; Sivaram et al., 2004; Bhattachary et al., 2007; Satish et al., 2008; Subashkumar et al., 2013).

In vitro studies on betel extract against pathogenic bacteria isolated from cultured fishes showed that the extract was able to inhibit the growth of *Vibrio alginolyticus*, *Vibrio vulnificus, Aeromonas hydrophila*, *Streptococcus* spp, *Photobacterium damsela* and *Micrococcus* sp. (Nik-Haiha et al., 2011). Similar results were obtained *in vitro* for Vibriosis caused by *Vibrio alginolyticus* in Asian seabass, *Lates calcarifer*, Motile Aeromonas Septicemia (MAS) caused by *Aeromonas hydrophila* in *Pangasius sutchii* and Nocardiosis in red snapper, *Lutjanus erythropterus* indicating the potential betel extract as alternative medication against bacterial diseases in fish (Nik-Haiha et al., 2014, Bond

& Senggagau, 2019 and Nik-Haiha et al., 2011). As for lemongrass extract, it was effective to be used as one of the therapeutic herbs against marine leech in hybrid grouper (Fadzilah & Azmi, 2018). Pathirana et al., (2019) demonstrated that lemongrass' oil possessed bactericidal activity against *Lactococcus garviae*, *Streptococcus iniae*, *Edwardsiella tarda* and *S. parauberis* isolated from olive flounders in Korea. Othman et al., (2018) suggested the best concentration of 100 mg/L of betel leaves crude extract as antimicrobial agent against marine bacteria.

Due to numerous reports on betel and lemongrass extracts on their antimicrobial properties, we carried out on the application of these two plant extracts in preventing or treating bacterial diseases in shrimps. The goal of the current study was to ascertain if betel leaves and lemongrass extracts could lower the prevalence of the *V. parahaemolyticus* bacteria causing AHPND in white shrimp.

Materials and Methods

Source of shrimps

Arca Biru Sdn Bhd in Kedah provided a total of 60 specified pathogen-free (SPF) white shrimps that had been in culture for 20 days. Using histopathology and an EMS-2 detection kit, the shrimps were evaluated and found to be AHPND-free. For the tests, only the prawn batches that tested negative were used. They were split into three groups (A, B, and C), with 30 white shrimps in group A (normal pellet), 15 in group B (normal pellet mixed with 100 ppm betel leaf), and another 15 in group C (normal pellet mixed with 200 ppm lemongrass). The shrimps were fed for 14 days, and then those in group A were further split into two groups, positive and negative control groups, in advance of the challenge tests.

Source of bacteria V. parahaemolyticus

Bacterial isolate identified as 3 HP, obtained from Shrimp-virus interaction laboratory (ASVI), CENTEX SHRIMP in Thailand was used for challenge test. Bacterial isolate was prepared from -80 °C glycerol-stock that was sub-cultured onto Trypticase Soy Agar medium supplemented with 1.5% NaCl. After overnight culture at 30 °C, 20 - 25 colonies of the bacteria were inoculated in Brain Heart Infusion broth and were shaken overnight at 30 °C to prepare the bacterial suspension for the challenge test.

Source of lemongrass and betel leaves

The products SitroPro® (PI 2017703131) and SirehMax® (MY-172900-A), which contain lemongrass and betel leaves, respectively, were both extracted in accordance with the prescribed procedures (FRI, 2022). Briefly, lemongrass was purchased from a local supplier in Johor and betel leaves were procured from Terengganu. Lemongrass was cleaned, chopped, dried at 40 °C for 12 - 24 hours and grinded using laboratory grinding machine. Approximately 200 g of powdered lemongrass was mixed with 1 L of ethanol (ratio 1: 5) and kept for 2 - 5 days at room temperature. The mixture was filtered through muslin cloth and the residues were adjusted to the required concentration with the extraction fluid for further extraction. An aliquot of extracted liquid was subjected to rotary evaporator for 3 - 4 h at 70 - 80 °C. The extraction liquids were stored at 4 °C in chiller for further usage. As for the betel leaves, the betel leaves were made into a powder using a blender after being dried for 3 to 7 days at room temperature (24 to 32 °C) in a shady area. A 100g of dried powdered betel leaves were immersed in 1 L ethanol (ratio 1:10) and kept for 2-5 days at room temperature. The extract obtained was then filtered, evaporated to dryness with a rotary evaporator and re-suspended in ethanol (90% concentration) to achieve a stock concentration of 100 mg/ml. The plants were stored in dark vials at 4 °C until further use. Both lemongrass and betel leaves used ethanol as type of solvents during the

extraction period.

Antibacterial assay

The antibacterial activity of the herbal extracts was qualitatively determined by disc diffusion method as previously reported by Bauer et al., (1966) and Anderson (1974). Briefly, the bacterial inoculum was spread evenly onto the surface of the Mueller Hinton agar plates and allowed to dry for 10 minutes. Sterile filter paper discs were impregnated with 100 µl of each extract (100 mg/ml) and later transferred onto the inoculated agar surface. Oxytetracycline, 30 µg/disc (Oxoid, UK) and Oxolinic acid, 2 ug/disc (Oxoid, UK) were used as positive control and the solvent (ethanol) as a negative control. Each extract was assayed in triplicate. All the plates were incubated at 37 ^{o}C for 24 h. The antibacterial activity was evaluated by measuring the zone expressed as millimeter (mm) of inhibition against test organism.

Preparation of herbal diets for experiment

The concentration used for betel leaves extract was 100 ppm while 200 ppm for lemongrass extract (Abdullah et al., 2018; Shaharah et al., 2022; Fadzilah et al., 2015 and Fadzilah et al., 2018). The extract was mixed with 50 ml of distilled water before being sprayed equally on 1 kg of pellet. For After 30 minutes of air-dry, the mixed pellets were packed into 2 g per packet and stored in chilled refrigerator. The shrimps were fed 5% of its bodyweight three times daily for 14 days.

Immersion challenge test

The challenge test was conducted by immersion of five shrimps for 1 minute in a 1 L aquaria tank containing *V. parahaemolyticus* suspension of bacterial density 1 x 10^8 cells/ml. After 1 minute, the shrimps were transferred into a 2 L aquaria tank contained 1 x 10^6 cells/ml bacterial suspension of *V. parahaemolyticus* and observed for the mortality within 24 hrs. The challenge test used in the present study was slightly modified from Tran et al., (2013) method.

Detection of AHPND by PCR

After 24 hours post infection, the hepatopancreas organ from alive shrimp from each group was dissected symmetrically into two parts; one for PCR while another for histology study. As for the PCR, the hepatopancreas were homogenized and inoculated on TSA plate for bacteria growth. The *V. parahaemolyticus* bacterial isolates from each group were then extracted for DNA using G-spin™ Total DNA Extraction Kit (iNtRON Biotechnology, Korea). The amount of template DNA in the 20 µl PCR reaction volume was in the range of 100-150 ng. AHPND primer set 1, (AP1) (Flegel & Lo, 2014) was used for the amplification of AHPND bacterial DNA fragments. The following are the sequences of the forward (F) and reverse (R) primer sets: AP1F, 5'- CCT TGG GTG TGC TTA GAG GAT G -3' and AP1R, 5'- GCA AAC TAT CGC. PCR was carried out using 0.2 ml microfuge tubes. The total volume reaction mixtures were 20.0 µl which contained 13.8 µl sterile distilled water, 2.0 µl 10 x PCR buffer without $MgCl_2$, 0.4 µl of 25 mM deoxyribonucleotide phosphates, 1. 2µl of 2 5mM $MgCl_2$, 0.2 µl of each primer, 0.2 µl of 500 units *Taq* DNA and 2µl template DNA. The cycling reactions were conducted for 5 min at 94°C, followed by 30 cycles of 30-sec denaturation at 94 °C, 30-sec annealing at 60 °C and 60-sec extension at 72 °C, plus a final 10 min extension at 72 °C (Eppendorf). Gel electrophoresis of 5 µL PCR products was done in 1.5% agarose gel at 400mA, 70V at 45 minutes. Stained gel was viewed under UV-illumination using Gel-DOC for band detection. The positive band for AHPND was confirmed at 700 bp.

Detection of AHPND by histology

The preparation of specimens for histology was done following Bell and Lightner (1988). The

shrimp from each group were injected with Davidson's fixative before fixing in Davidson's solution for 24 - 48 hours. After 24 - 48 hours in Davidson's solution, the specimens were immersed in 70% ethanol until the processing date. The specimens were processed by an automatic tissue processor (Leica ASP 300) and embedded in paraffin wax, which was then sectioned at 5 micrometers, stained with Haematoxylin and Eosin (H & E), and finally mounted with DPX before being examined under a compound microscope (Leica DM5000B) connected to a digital camera (Leica DFC 320) associated with computer software (Leica QWin). The pathology confirmation of AHPND was described as sloughing of hepatopancreatic tubule epithelial cells, prominent karyomegaly, and melanized granulomas (Lightner, 1996; Leano & Mohan, 2012).

Statistical Analysis

A One-Way ANOVA was performed to determine the significant differences for the survival of shrimp after challenged with bacteria *V. parahaemolyticus* that caused AHPND in the group received herbs diet and without. All statistical analyses were executed at the significant level of 0.05 using the statistical program SPSS Statistic, Version 20.

Results and Discussion

The antibacterial activity of the ethanolic extract of betel leaves exhibited moderate antimicrobial effects on test organism indicated by a moderate (10 - 14 mm) zone of clearance (Fig.1). While methanolic extract of lemongrass showed weak activity against bacteria. The inhibitor zone observed for betel leaves was similar with reference antibiotics (oxolinic acid) discs but less compared with antibiotic oxytetracycline (Fig.1). As for the lemongrass extract, the inhibitor zone was less than both antibiotics as well as betel leave extract. The antibacterial assay results showed that extraction from betel leaves has a moderate positive antibacterial activity toward *V. parahaemolyticus* while a weaker antimicrobial activity for lemongrass extract. The betel leaves extract having the antimicrobial activity showed in the present study was also reported by Veronica and Julian (2013). In their study, the crude extract of betel was able to inhibit *V. parahaemolyticus* ATCC 17802. Studied on the inhabitation activity demonstrated in betel leaves extract human pathogen were reported due to fatty acids and hydroxychavicol component (Ramji et al., 2002 and Bhattacharya et al., 2007). Both components were able to exhibit antibacterial activity by targeting the structure and function of bacterial cell walls and membranes (Subashkumar et al., (2013). The same authors also highlighted that crude extracts of betel leaves contain one or more of the phytochemical compounds (sterol, chavicol, and tannin) which have inhibitory effects on the bacteria.

In the present study, the lemongrass extract showed weaker antimicrobial activity. Suree and Pana (2013) reported that lemongrass extract was active against only 17 strains (7 - 11 mm) of the 25 strains in human pathogenic bacteria. Behboud et al., (2012) reported that antibacterial effect of lemongrass was most significant against Gram positive bacteria compared with Gram negative bacteria. In the present study, the challenge bacteria *V. parahaemolyticus* was a Gram negative bacterial and this may be the contributing factor to the weaker antibacterial activity.

The results of the *V. parahaemolyticus* after 24-hour challenge test showed a higher survival rate of 87.5% (average 3.5 ± 0.71) in shrimp given betel leaves extract compared to 50% (average 2.5 ± 0.00) in the positive control group and 37.5% (average 1.5 ± 0.71) in the lemongrass group (Table 1). No mortality was seen in negative control shrimp. One-way Anova analysis showed there was a significant difference ($p < 0.05$) in survival between the groups. Tukey HSD analysis showed that two groups of survival were observed; in which the first group consisted of shrimp from negative and those

fed with betel leaves extract while the second consisted of shrimps from positive and those received lemongrass extract. *V. parahaemolyticus* bacteria was detected in all the groups except in negative control group after 24 hours observation using AP1 primer. Sloughing of hepatopancreatic tubule epithelial cells, prominent karyomegaly, and melanized granulomas were observed under histological sections and validated as characteristics diagnosis for AHPND. Positive pathology AHPND was also seen in hepatopancreatic organ of all the groups except shrimp from negative control group (Fig.2). Both positives AHPND detection either from PCR detection or histopathology further confirmed the mortality in the present study was due to bacteria *V. parahaemolyticus.*

The higher survival rate seen in shrimp fed with betel leaves extract could indicate that they have a better defense system against bacteria *V. parahaemolyticus*. Nalina and Rahim (2007) showed that crude aqueous extract of *Piper betle* leaves showed antibacterial effect against *Streptococcus mutans* while Subashkumar et al., (2013) showed its antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. On the other hand, shrimp fed with lemongrass extracts did not show any significant results. Results of betel leaves and lemongrass extract in the present study were not able to be compared with other study due to unavailability of report on application of these herbs in shrimps. However, there are some reports on using others herbs extraction for prevention of bacterial infection in farmed shrimp. A better resistance against *V. harveyi* in shrimps fed with 25 mg/kg feed of turmeric extract was reported by Vanichkul et al., (2010). One of the limitations of this study is the small number of shrimps used in each replicate. This is because we want to make sure the shrimp have enough food and space to avoid cannibalism. We believe that, shrimp fed a diet containing betel leaves extract exhibit resistance to *V. parahaemolyticus*. The present study showed that *Piper betle* leaves extract has a good potential for prevention and treatment of bacterial diseases particularly in preventing AHPND outbreak in shrimp. Betel leaf has low toxicity and is safe for *P. vannamei*, Furthermore, it is friendly to the environment and it will help farmers to minimize losses due to AHPND.

Table 1: Average survival (%) of shrimp (14-Days oral dietary) after challenged with pathogenic *V.parahaemolyticus* that caused AHPND

Group	**Average survival (%) of post challenge shrimp**	
	0 hour	**24 hours***
Control negative	100.0 ± 0.0	100.0 ± 0.0 [a]
Control positive	100.0 ± 0.0	50.0 ± 0.0 [b]
Betel leaves extract	100.0 ± 0.0	87.5 ± 17.7 [a]
Lemongrass extract	100.0 ± 0.0	37.5 ± 17.7 [b]

*Note: *One-way Anova test on differences between means of survival of shrimp between groups showed a significant difference ($p < 0.05$). Superscript indicates the post hoc group by Tukey HSD analysis.*

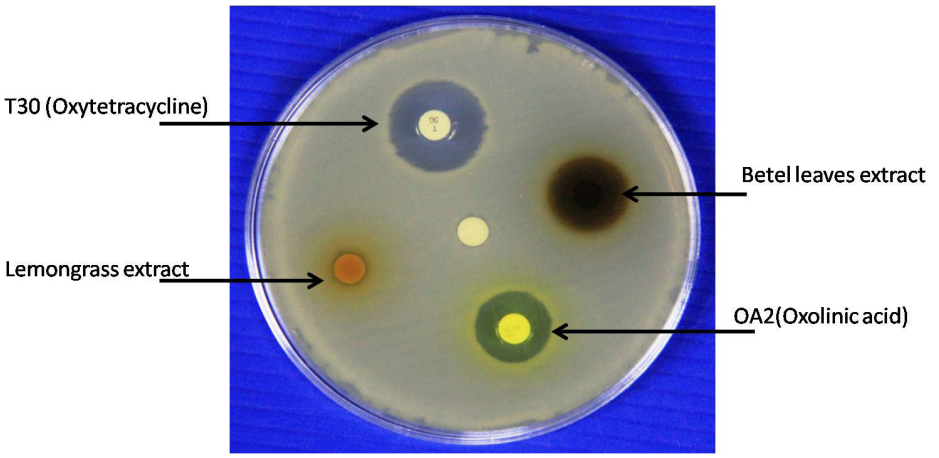

Figure 1: Results of antibacterial activity of betel leaves extract, lemongrass extract, oxolinic acid and oxytetracycline obtained using disc diffusion method of 24 hours *V. parahaemolyticus* grown cultures

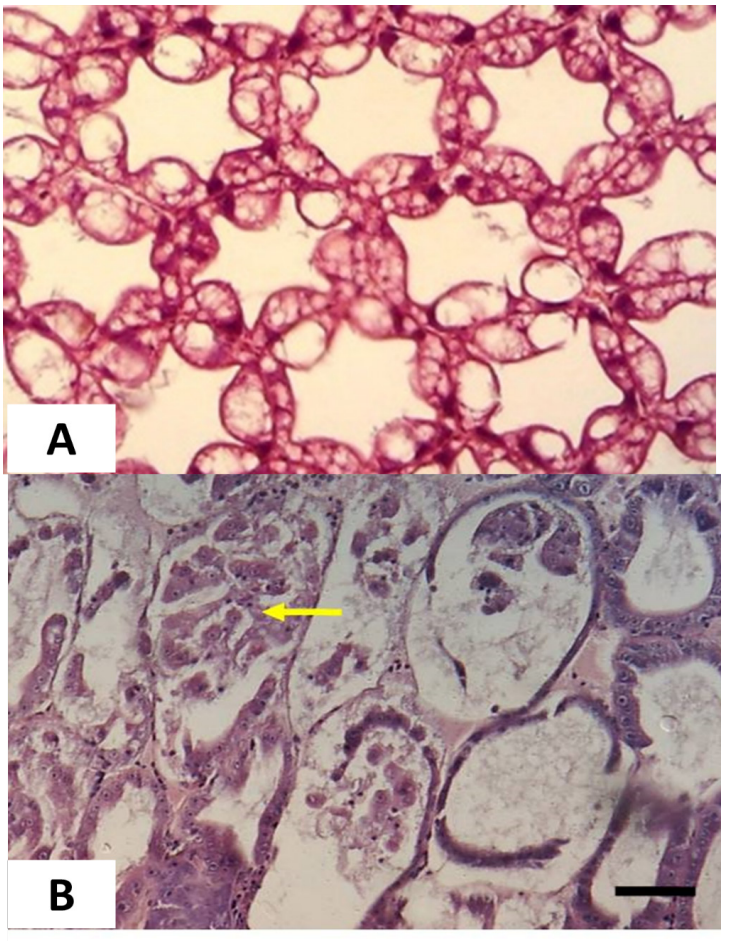

Figure 2: Histopathological finding from the hepatopancreas (HP) organ of post infection shrimps. No sloughing of epithelial cells from HP tubule was seen in negative shrimps (A) and sloughing of epithelial cells from HP tubule (arrow) were observed in all shrimps challenged with *V. parahaemolyticus*. H&E, Scale bars (A & B) = 50 µm

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