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Research Article

Cloning and Characterization of Two Inhibitors of Apoptosis (IAP) from *Eriocheir hepuensis* and Their Expression Pattern in the Immune Organs under Azadirachtin Stress

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Inhibitors of apoptosis proteins (IAPs) maintain a balance between cell growth and cell death by suppressing caspase activity and modulating immunological responses. Here, two IAPs (EhIap1 and EhIap2) were identified from *Eriocheir hepuensis* using RT-PCR technology and characterized using bioinformatics software. Sequence analysis showed that the open reading frames of EhIap1 and EhIap2 were composed of 1,839 bp and 696 bp and encoded 612 amino acids and 231 amino acids, respectively. EhIap1 contained two BIR domains, one UBA domain, and one RING domain, and EhIap2 only contained two BIR domains. qRT-PCR showed that EhIap1 and EhIap2 were expressed in various tissues, and their expression levels were highest in the hepatopancreas. Expression levels of EhIAP1 and Eh1ap2 were more than 87.09 and 411 times higher in the hepatopancreas than in muscle, respectively. Under azadirachtin stress, the expression of EhIap1 and EhIap2 was increased in three immune tissues (hepatopancreas, gill, and heart). The expression of EhIap1 was 18.44, 17.75, and 6.16 times higher in hepatopancreas, heart, and gill tissues under azadirachtin stress compared with the control group, respectively. The expression of EhIap2 was 18.44, 15.48, and 3.91 times higher in hepatopancreas, gill, and heart tissue under azadirachtin stress compared with the control group, respectively. The expression of EhIap1 and EhIap2 was higher in surviving individuals than in dead individuals. Overall, EhIap1 and EhIap2 genes were first cloned, and our findings indicate that EhIAPs might be involved in *E. hepuensis* against azadirachtin through regulating apoptosis.

1. Introduction

Eriocheir hepuensis (Crustacea: Decapoda: Brachyura: Varunidae) is a mitten crab peculiar to the Beibu Gulf that was named after the county in which it was initially discovered (Hepu County). It has been documented in the Nanliujiang River, Qinjiang River, and Maolingjiang River in southern Guangxi Province [1].

Apoptosis is an important biological process that plays a key role in regulating growth, development, and immune responses. Faulty regulation of apoptosis can result in excessive cell proliferation or cell death, which can lead to disease. Inhibitor of apoptosis protein (IAP) is a highly conserved endogenous inhibitor of apoptosis that was first identified in baculovirus in 1993 [2]. It was later found to widely occur in viruses, bacteria, yeast, insects, and mammals. Five families of IAPs have been identified to date: X-linked IAP (XIAP), c-IAP1, c-IAP2, NAIP, and Survivin. IAPs possess at least one baculovirus inhibitor protein (BIR) repeat domain [3], which can interact with apoptosis protease (caspases) and inhibit its activity; the RING domain in the C-terminus is thought to have a ubiquitination function [4]. An IAP protein containing three BIR regions cloned from *Penaeus monodon* [5] negatively regulates the apoptosis protease. In *Drosophila melanogaster*, apoptosis is mainly controlled by DIap1 proteins [6]. Insect SF-9 cell

lines can also express a similar protein that can inhibit reaper-induced apoptosis [5].

Azadirachtin is thought to be an excellent pesticide because of its low toxicity; it is thought to be nontoxic to humans and other mammals (Authority et al., 2018). Azadirachtin is often used in aquaculture and fisheries to kill parasites on aquaculture animals or remove harmful bacteria from aquaculture water [8]. It has even been used as an oral medicinal agent for *Carassius auratus* to provide resistance to pathogenic microorganisms [9]. Pesticide residues have detrimental effects on organisms, especially on the wild germplasm resources of aquatic organisms [10]. When azadirachtin is administered in low doses, it induces apoptosis, but high concentrations of azadirachtin are toxic to individuals and induce excessive cell death [11].

Clarifying the toxic effects of azadirachtin on *E. hepuensis* is extremely important because it is a commonly farmed aquatic arthropod. Preliminary laboratory studies have shown that the LC50 of azadirachtin for *E. hepuensis* is 57 mg/L; relevant oxidation indexes have also been determined by Liao et al. [12]. However, studies of the effects of azadirachtin on apoptosis have not yet been conducted. In this study, the open reading frames of two IAPs (EhIap1 and EhIap2) in *E. hepuensis* were cloned and characterized by bioinformatics analysis, and their amino acid structures were predicted. Acute toxicological expression profiles of EhIap1 and EhIap2 in immune-related tissues (hepatopancreas, gill, and heart) under azadirachtin stress were determined. The results of this study provide new insights into the mortality rate of *E. hepuensis* under azadirachtin stress and have important implications for the protection of its germplasm resources.

2. Materials and Methods

2.1. Temporary Culture of Crabs and Azadirachtin Challenge. Individuals of *E. hepuensis* (average weight 13 ± 1.5 g) were acquired from Dongfeng Seafood Market in Qinzhou, Guangxi Province, PR China. After crabs were incubated for 3 d with tap water at 20–22°C, pH 7.5–8.0, DO 6.8–7.2 mg/L, and salinity 0.1–0.3 and on a diet of fresh squid meat, individuals of the same size and with intact appendages were placed into 40-L plastic square barrels ($45 \times 30 \times 30$ cm).

Azadirachtin emulsifiable concentrate (0.3%) was bought from Chengdu Lvjin Biotechnology Co., Ltd.. The Lc50 of 48 h of azadirachtin for *E. hepuensis* was 57.00 mg/L. However, water was less transparent in the aquaculture tank when the concentration of azadirachtin exceeded 20 mg/L, thus, we used azadirachtin at a concentration of 20 mg/L in the experimental group for the convenience of observation [13]. Thus, in the azadirachtin challenge experiment, 20 mg/L azadirachtin was administered to 80 crabs in the stress group, and the control group consisted of 40 crabs without azadirachtin. The density of the stress and control group was 10 crabs per barrel.

2.2. Samples Gathering, RNA Extraction, and Reverse Transcription. Various tissues, including muscle, intestine, heart, stomach, hepatopancreas, gills, gonad, and hemolymph, of the crabs were collected and rapidly frozen in liquid nitrogen. To determine the concentration of total RNA of *E. hepuensis* extracted by the TRIzol method, we measured the absorbance at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and RNA integrity was assessed by electrophoresis. Samples were stored at −80°C for subsequent experiments. TransScript Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal, TransGen Biotech, Beijing, China) was used to perform first-strand cDNA synthesis and gDNA removal. The manufacturer's protocol was used.

2.3. Cloning of the EhIap1 and EhIap2 Gene. The coding portions of EhIap1 and EhIap2 were cloned using reverse transcription-polymerase chain reaction (RT-PCR). Two pairs of specific primers were designed based on unpublished transcriptome data for *E. hepuensis* using Oligo 6.0 software: EhIap1-F, EhIap1-R, EhIap2-F, and EhIap2-R (Table 1), to amplify the open reading frame (ORF) of EhIap1 and EhIap2. Blue and white spots on the plate medium were screened, and PCR was used to detect recombinant bacteria after the amplified product was grafted into the pEASY®-Blunt Simple Cloning Vector and transfected into Trans1-T1 Phage Resistant Chemically Competent Cells (TransGen Biotech, Beijing, China). A plasmid containing the inserted EhIap1 and EhIap2 fragments was extracted from cultured *E. coli* and used for DNA sequencing with universal primers by Sangon Biotech (Shanghai, China).

2.4. Bioinformatics Sequence Analysis. The Expert Protein Analysis System (EXPASY) server (http://web.expasy.org/protparam/) was used to analyze the amino acid composition, molecular weight, and isoelectric point. Alignment of cognate sequences was performed using the BLAST program on the NCBI (https://www.ncbi.nlm.nih.gov/). Subcellular localization was predicted using Softberry (http://linux1.softberry.com/berry.phtml?%20topic=protection&group=programs&sugarout=process/). Motif Scan (https://myhits.sib.swiss/cgi-bin/motif%20scan) and Protter (http://wlab.ethz.ch/protter/#/) were used to locate and analyze protein functional sites. Multiple sequence alignments were performed using the Clustal X 2.0 program. Phylogenetic analysis was conducted using the neighbor-joining method in MEGA 7.0 with a minimum of 1,000 bootstrap replicates. SWISS-MODEL (https://swissmodel.expasy.org/) was used to build tertiary structures, and comparisons were made using PyMOL software.

2.5. Expression Analysis. To examine the expression patterns of EhIap1 and EhIap2 in different tissues on a qPCR system (USA, BIO-RAD CFX Connect), we used $2 \times$ PerfectStart Green qPCR SuperMix (TransGen Biotech, Beijing, China) with previously synthesized cDNA (50 ng/μL) to amplify the EhIap1 and EhIap2 fragments using the primers (EhIap1DL, EhIap2DL) shown in Table 1. Real-time qPCR was

Table 1: Primer sequence.

Primer name	Forward	Reverse	Amplification length (bp)	Tm (°C)
EhIap1	CTGCTGCCAGACGCTAGT	ATATGATGTCCCCGCCTT	2190	56
EhIap2	CGCCCGTGAAGGAATACAT	TTGAGTGCCCGTTGTTCG	1080	56
Ef1-DL	TCTGACTCCAAGAACGACCC	CAGGCAATGTGAGCAGTGTG	131	60
EhIap1DL	TCCACTCCCACCAATCCAG	GCCACCTTCTCCATCACAG	110	60
EhIap2DL	ACCCGCAGTTCAACACAGAA	GTAAAAGAAGCCAGCCGTCA	104	60

conducted per the manufacturer's instructions, and β-actin was used as the internal reference gene.

After azadirachtin stress, crabs were anesthetized on an ice plate for 5–10 min before being sampled. Immune organs (including hepatopancreas, heart, and gill) of *E. hepuensis* were collected from three crabs 3, 6, 9, 12, 24, and 48 h after exposure to azadirachtin, and RNA was extracted from these tissues and used in qPCR analysis.

2.6. Statistical Analysis. All data in this study were expressed as the mean ± standard deviation ($n = 3$). Statistical significance was assessed by one-way analysis of variance (ANOVA) using SPSS Statistics 26 software. An independent *t*-test was conducted to compare the number of surviving crabs in the experimental and control groups. Significant differences between groups were assessed using Duncan's method ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$).

3. Results

3.1. Cloning and Identification of EhIap1 and EhIap2. The TRIzol method was used to extract and purify total RNA from *E. hepuensis* tissue (Figure 1(a)). Three stripes of RNA were assessed by electrophoresis: 28S, 18S, and 5S. Sequences of 2,190 bp of EhIap1 (GenBank accession number: ON332510) and 1080 bp of EhIap2 (GenBank accession number: ON332511) were produced by PCR amplification (Figures 1(b) and 1(c)).

3.2. Sequence Analysis and Characteristics of the EhIap1 and EhIap2. The molecular formula of EhIap1 was $C_{2927}H_{4621}N_{807}O_{931}S_{36}$, and the most abundant amino acid was serine (10.6%), followed by alanine (9.0%); the least abundant amino acid was tryptophan (1.1%). EhIap1 had a predicted molecular mass of 67.16 kDa and an isoelectric point of 5.48. The molecular formula of EhIap2 was $C_{1190}H_{1726}N_{336}O_{335}S_9$, and the most abundant amino acid was proline (9.5%), followed by glycine (9.2%); the least abundant amino acid was lysine (1.3%). EhIap2 had a predicted molecular mass of 26.38 kDa and an isoelectric point of 6.04. The absence of a signal peptide was predicted in both EhIap1 and EhIap2. EhIap1 and EhIap2 were predicted to be localized to the cytoplasm.

Comparative analysis by BLAST revealed that the similarity in the nucleotide sequence of EhIap1 with that of *Procambarus clarkii*, *Scylla paramamosain*, *Penaeus monodon*, *Litopenaeus vannamei*, and *Homarus americanus* was 73.39%, 72.61%, 68.32%, 68.06%, and 66.23%, respectively. EhIap1 was predicted to contain two signature domains of the IAP family: the BIR repeat domain (54–118, 198–263), an UBA-like SF domain (396–433), and a RING-HC-BIRC2/3/7 domain (565–600) (Figure 2(a)). EhIap1 was predicted to contain one N-glycosylation site (^{26}NITG^{29}), two tyrosine kinase phosphorylation sites (^{254}RWYPECVY^{261} and ^{273}KGKK^{281}), two N-myristoylation sites (^{92}GIVGAW^{97} and ^{415}GAVSSS^{420}), nine protein casein kinase C phosphorylation sites (^{8}TPK^{10}, ^{20}SLR^{22}, ^{39}TKK^{41}, ^{47}SLR^{49}, ^{102}TPR^{104}, ^{212}TPK^{214}, ^{363}TLR^{365}, ^{368}SAR^{370}, and ^{391}SER^{393}), and ten protein kinase C phosphorylation sites (^{20}SLRE^{23}, ^{57}TFID^{60}, ^{102}TPRE^{105}, ^{162}SYPE^{165}, ^{212}TPKE^{215}, ^{301}TESE^{304}, ^{363}TLRE^{366}, ^{433}SQHD^{436}, ^{440}SNTE^{443}, and ^{461}SQAD^{464}) (Figures 2(a) and 2(b)).

The similarity of the EhIap2 sequence of *E. hepuensis* with that of *Portunus trituberculatus*, *Eriocheir sinensis*, *Homarus americanus*, *Procambarus clarkia*, and *Penaeus japonicus* was 77.59%, 68.45%, 67.77%, 66.84%, and 65.47%, respectively. EhIap2 was predicted to contain two BIR repeat domains (5–50 and 117–182) (Figure 2(c)). EhIap2 was predicted to contain three casein kinase II phosphorylation sites (^{99}TQAD^{102}, ^{120}TFRE^{123}, and ^{210}SRQE^{213}), one cAMP-PHOSPHO-SITE protein kinase phosphorylation site (^{117}RPQT^{120}), three N-myristoylation sites (^{54}GLPTGN^{59}, ^{156}GGLFAW^{161}, and ^{223}GQEAGW^{228}), and five protein kinase C phosphorylation sites (^{48}SCR^{50}, ^{69}TGR^{71}, ^{86}STR^{88}, ^{120}TFR^{122}, and ^{185}TGK^{187}) (Figures 2(c) and 2(d)).

3.3. Phylogenetic Tree Building and Amino Acid Sequence Comparison. In the phylogenetic tree that was constructed with their amino acid sequences, EhIap1 was clustered with *P. trituberculatus*, and EhIap2 was clustered with *E. sinensis* first, then formed a group with other crustaceans IAPs separately, and IAPs from vertebrates were clustered with insecta into one bough. EhIap1 protein of had 8 motifs (7, 2, 10, 4, 1, 8, 6 and 3 in order) and missed motifs 5 and 9 compared with other crustaceans. EhIap2 protein had the least numerous motifs (2, 10, 7, and 1 in order) compared with other crustaceans (Figure 3).

The three-dimensional structures of EhIap1 and EhIap2 were shown in Figure 4. The result reflected a similarity between EhIap2 and EsIap, which had secondary structural order of $\alpha1\alpha2\beta1\beta2\beta3\alpha3\alpha4\alpha5\alpha6\alpha7\alpha8\alpha9\alpha10\alpha11\alpha12\alpha13\beta4\beta5$, considered as BIR domains that contain the signature sequence $CX_2CX_{16}HX_6C$ (C, cysteine; H, histidine; and X, any amino acid) and fold as three-strandedβ sheets surrounded by four α-helixes, which was considered as bind to caspase 3/7.

3.4. Relative Expression of EhIap1 and EhIap2 Genes in Normal Tissues. As determined by real-time PCR detection,

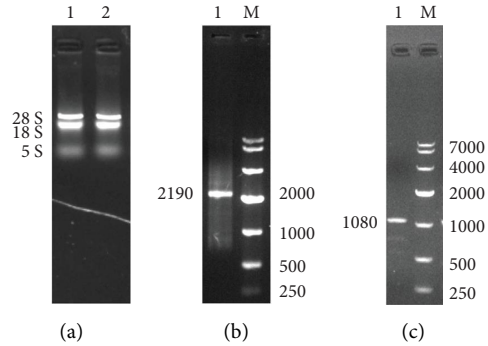


Figure 1: Electrophoresis map of *E. hepuensis* RNA (a), EhIap1 gene (b) and EhIap2 gene (c). M: 10000 bp DNA marker.

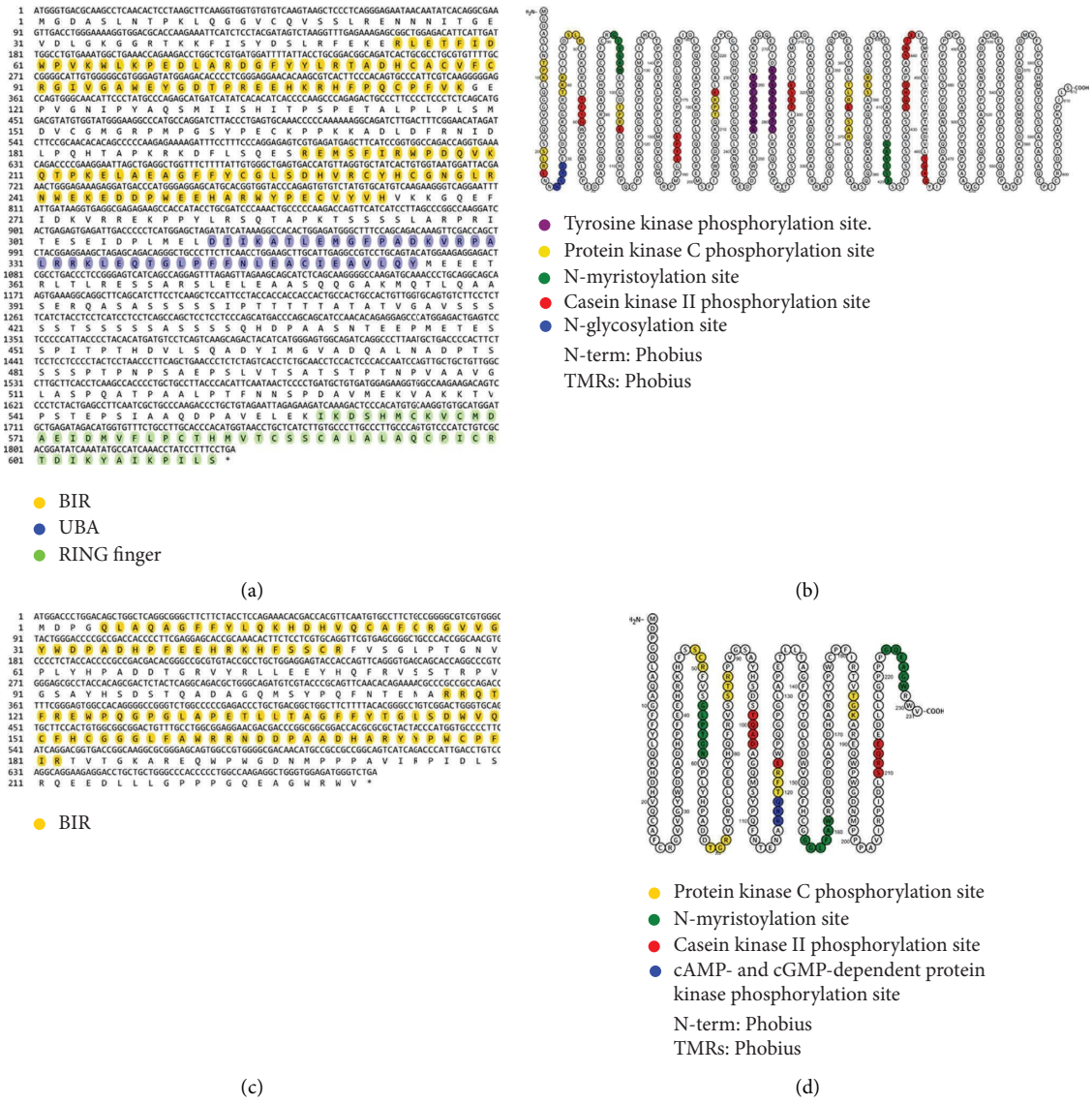
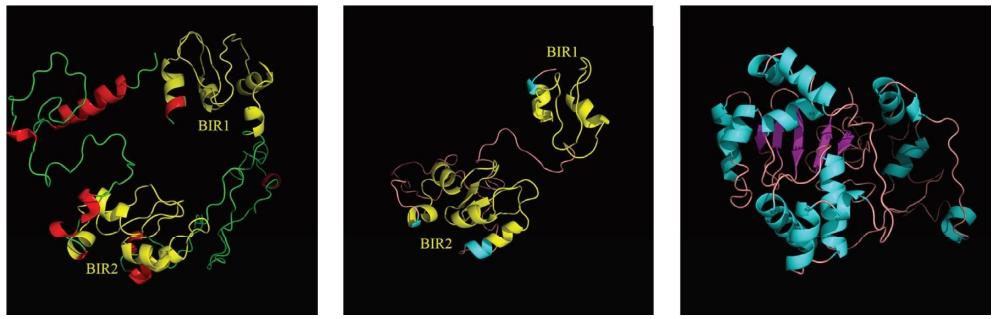


Figure 2: Nucleotide sequences, amino acid sequences and functional sites of EhIap1 (a and b) and EhIap2 (c and d) in *E. hepuensis*.

qRT-PCR resulted in single products (Figure 5(a)), which suggested that the primers for the EhIap1 and EhIap2 genes were appropriate for QRT-PCR. The expression of EhIap1 and EhIap2 genes in different normal tissues was detected using the real-time fluorescence quantitative technique. EhIap1 and EhIap2 genes were highly expressed in all tissues, including muscle, intestine, heart, stomach, hepatopancreas, gill, gonad, and hemolymph, and their expression was highest

Figure 3: Neighbour-joining phylogenetic tree based on two IAP genes nucleotide sequences among some species.



(a) (b) (c)

Figure 4: Prediction of the tertiary structure IAP genes amino acid sequence. (a): EhIap1; (b) EhIap2; (c): Protein tertiary structure of IAP in *E. sinensis*.

in hepatopancreas, gill, and heart tissue. The expression of EhIap1 was more than 87.09 times higher in hepatopancreas than in muscle ($p < 0.01$), and the expression of EhIap2 was more than 411 times higher in hepatopancreas than in muscle ($p < 0.01$). The expression of EhIap1 in hemolymph and gill tissue was not significantly different ($p > 0.05$); the expression of EhIap2 was significantly higher in hemolymph than in gill tissue (Figures 5(a) and 5(b)).

3.5. The Expression Pattern of the EhIap1 Gene in Immune Organs under Azadirachtin Stress. The expression of EhIap1 in immune tissues (hepatopancreas, gill, and heart) changed following exposure to azadirachtin. The expression of EhIap1 was unimodal in hepatopancreas and heart tissue (Figures 6(a) and 6(e)). The expression of EhIap1 was stable in the first 12 h ($p > 0.05$) and rapidly up-regulated at 24 and 48 h, peaking at 48 h (18.44-fold in the hepatopancreas,

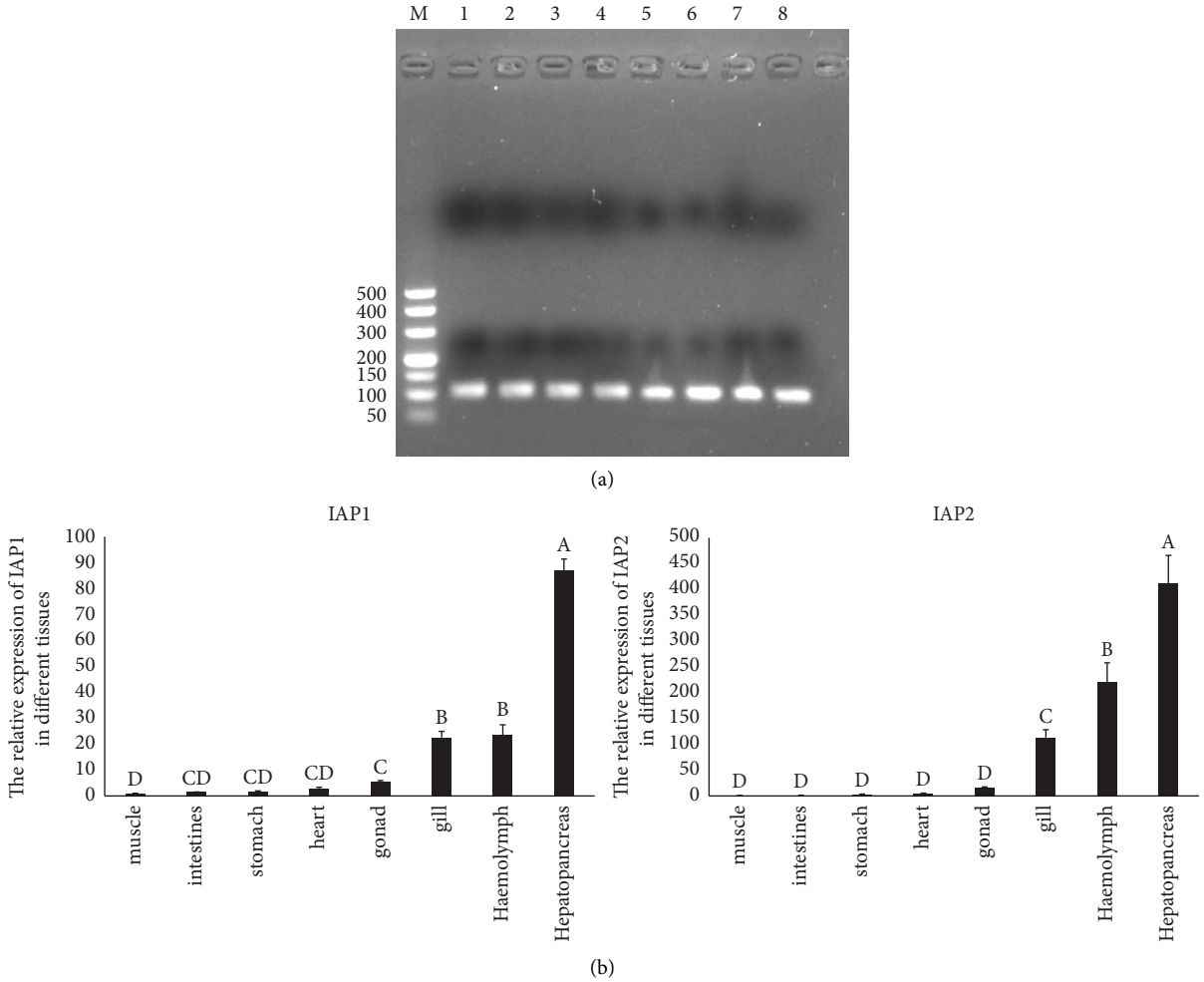


Figure 5: Verification of qRT-PCR primers of two IAP genes (a) and relative expression of two IAP genes in different tissues of normal *E. hepuensis* (b). The results are based on three parallel experiments and shown as meanvalues ± SD. Letters indicate significant differences.

17.75-fold in the heart, $p < 0.01$). The expression of EhIap1 was bimodal in gill tissue (Figure 6(c)); it was increasingly up-regulated at 6 h (6.16-fold, $p < 0.01$) and then down-regulated, but its expression remained higher in the treatment group than in the control group. Its expression began to be gradually up-regulated at 48 h (5.41-fold, $p < 0.01$). The relative expression of EhIap1 was 30.66, 14.59, and 5.41 times higher in the heart, gill, and hepatopancreas tissues of dead individuals than in control heart, gill, and hepatopancreas tissue, respectively. The expression of EhIap1 was higher in the hepatopancreas but lower in gill and heart tissue in surviving individuals compared with dead individuals (Figures 6(b), 6(d), and 6(f)).

3.6. The Expression Pattern of the EhIap2 Gene in Immune Organs under Azadirachtin Stress. The expression of EhIap2 in immune tissues (hepatopancreas, gill, and heart) changed following exposure to azadirachtin. The expression of EhIap2 was unimodal in hepatopancreas, gill, and heart tissue (Figure 7). In hepatopancreas, the expression of EhIap2 was rapidly up-regulated at 12 and 24 h and peaked at 24 h (18.44-fold, $p < 0.01$) (Figure 7(a)). In gill tissue, the expression of EhIap2 was rapidly up-regulated at 6 h (15.48-fold, $p < 0.01$) (Figure 7(c)) and then gradually decreased; and in heart tissue, the expression of EhIap2 was rapidly up-regulated at 24 and 48 h and peaked at 48 h (3.91-fold, $p < 0.01$) (Figure 7(e)). The expression of EhIap2 was 2.06, 4.79, and 2.74 times higher in the heart, gill, and hepatopancreas tissue of dead individuals than in control heart, gill, and hepatopancreas tissue, respectively. The expression of EhIap2 was higher in the hepatopancreas, gill, and heart tissue of surviving individuals than in dead individuals (Figures 7(b), 7(d), and 7(f)).

4. Discussion and Conclusions

E. hepuensis mitten crab has a congenital nonspecific immune system, and cell apoptosis is critically important for its ability to resist pathogens. IAPs maintain a balance between cell growth and cell death by suppressing caspase activity through their BIR structure and play a crucial role in numerous physiological processes, including homeostasis [14], tissue development [15], and immunological responses [16]. Azadirachtin, a class of highly oxidized limonoid-like substances belonging to tetracyclic

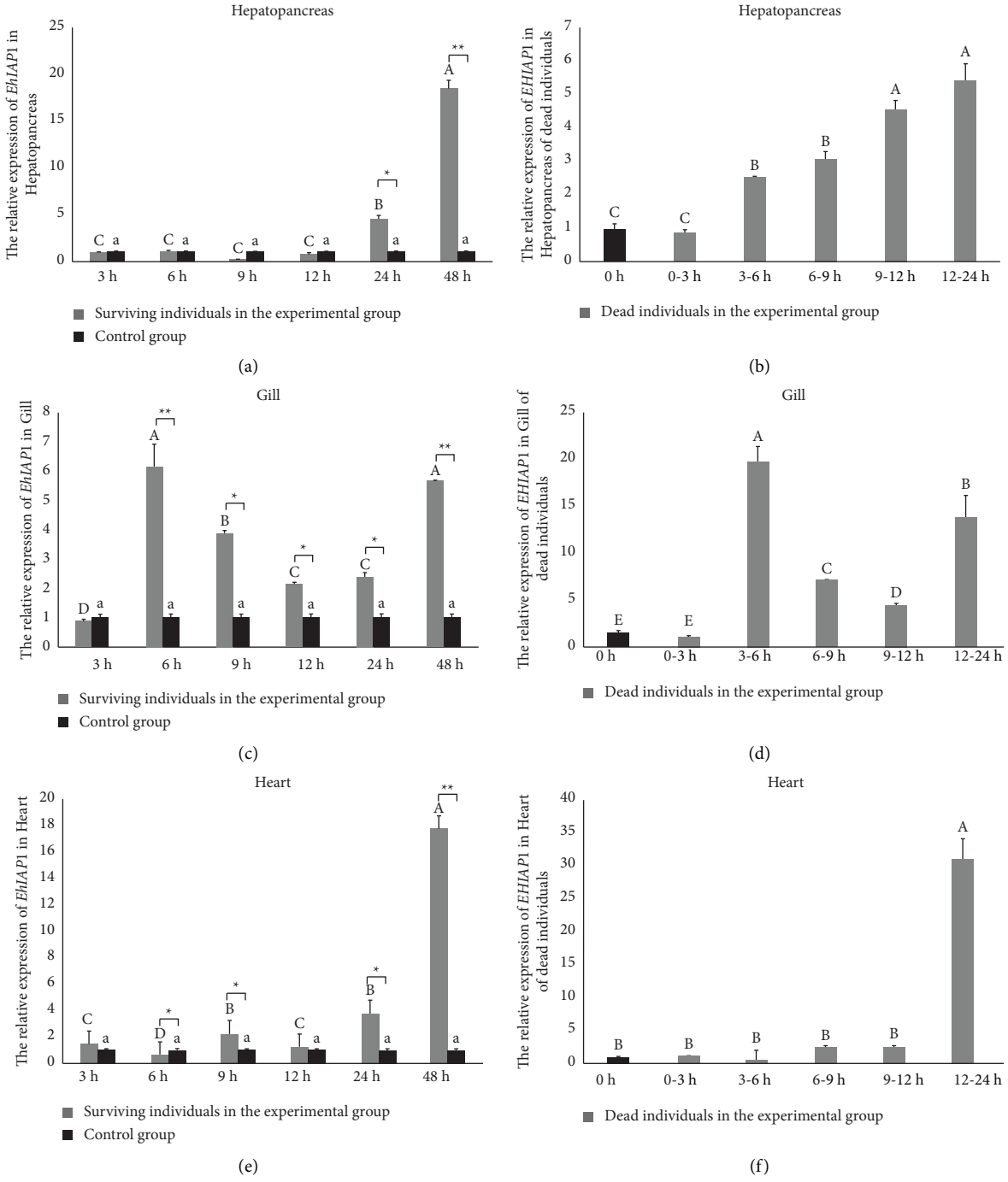


Figure 6: Expression of the EhIap1 gene in immune organs in control group and surviving individuals of experience group ((a) in hepatopancreas, (c) in gills, (e) in hearts) and dead individuals of experience group ((b) in hepatopancreas, (d) in gills, (f) in hearts) under 20 mg/L azadirachtin stress. The results are based on three parallel experiments and shown as mean values ± SD. Letters indicate significant differences. ($^{*}p < 0.05$, $^{**}p < 0.01$).

triterpenoids and mainly extracted from Neem seeds [17], has been shown to protect the pancreatic cells from apoptosis by inducing the autophagy signals [18], and its concentration for killing insects in citrus trees and locusts in grasslands is 5.0–7.5 mg/L and 18–25 mg/L, respectively [12]. Azadirachtin can not only control parasites on fish but also harm fish. Wu and Zhu [19] reported that the safe dose of azadirachtin for fish is 2.3 mg/L, and its safe dose for *E. hepuensis* obtained from our previous laboratory data is 3.5 mg/L [13]. Because this concentration for killing insects and locusts is higher than the safe dose for *E. hepuensis*, the application of azadirachtin in water poses a risk to the germplasm resources of *E. hepuensis*.

Azadirachtin can induce apoptosis in *Spodoptera frugiperda* Sf9 cells [20], but little is known about its effects on apoptosis signals of *E. hepuensis*. In this study, two novel IAPs with the typical domains of the IAP family were

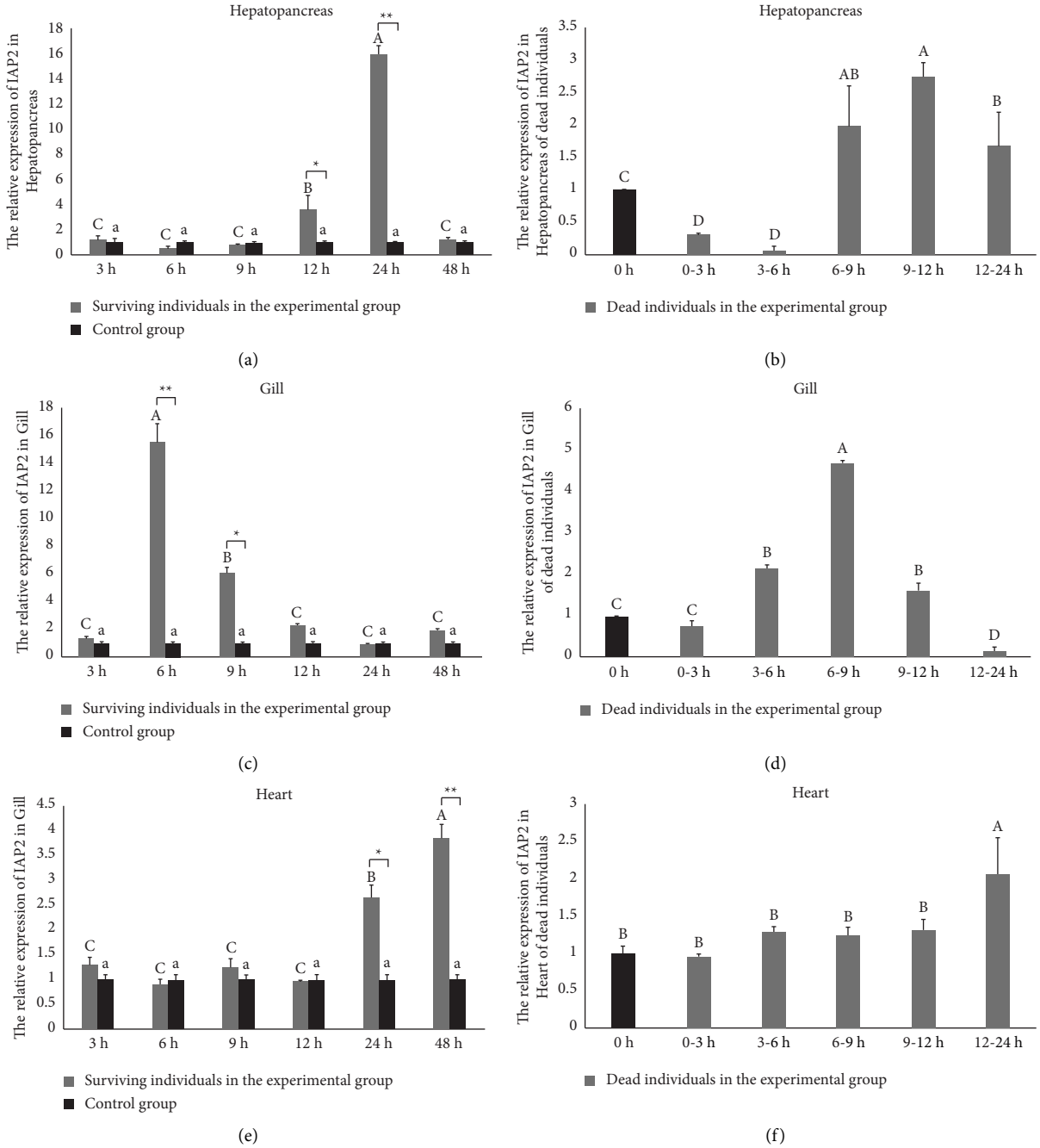


FIGURE 7: Expression of the EhIap2 gene in immune organs in control group and surviving individuals of experience group ((a) in hepatopancreas, (c) in gills, (e) in hearts) and dead individuals of experience group ((b) in hepatopancreas, (d) in gills, (f) in hearts) under 20 mg/L azadirachtin stress. The results are based on three parallel experiments and shown as mean values ± SD. Letters indicate significant differences. ($^{*}p < 0.05$, $^{**}p < 0.01$).

identified in *E. hepuensis* (EhIap1 and EhIap2). EhIap1 has the same conserved UBA domain and one RING domain, EhIap2 has only two BIR domains. BIR domains are typically made up of four alpha helices and a three-strandedβ-sheet, are conserved among IAPs and are present in multiple copies, being classified in type I and type II. Nevertheless, BIRs evolved different strategies to promote IAP prosurvival role. Type I BIRs are involved in protein-protein interactions, which in turn regulate the NF-κB pathway. Type II BIRs display a functional IBM groove for the direct binding of peptides, which provided the basis for SM design [21]. The spacing of cysteine and histidine residues (Cx2 Cx6 Wx3 Dx5 Hx6 C) in EsIAP1 was consistent with the other identified BIR2 domains; this might have contributed to the formation of a novel zinc-binding structure [22]. EhIap1 was clustered with PtIap, and EhIap2 was clustered with EsIap in the phylogenetic tree. These findings indicate that EhIap1 and EhIap2 are members of the IAP family in crustaceans.

To explore the function of EhIap1 and EhIap2, we characterized its expression in normal tissues and three immune organs following exposure to azadirachtin stress. EhIap1 and EhIap2 mRNA transcripts were detected in all analyzed tissues, including hepatopancreas, hemolymph, gill, gonad, heart, stomach, intestine, and muscle. CgIAP transcripts were detected in numerous tissues of the oyster *C. gigas* [23]. The expression patterns of EhIap1 and EhIap2 were similar in healthy tissues in *E. sinensis*, and the expression levels of these genes were highest in hepatopancreas, followed by hemolymph and gill tissue [24]. The high expression of EhIap1 and EhIap2 in the hepatopancreas indicates that the hepatopancreas might be the main organ of *E. hepuensis* responsible for mediating resistance to infection via cell apoptosis (B et al., 1998).

Following exposure to azadirachtin stress, the expression of EhIap1 was unimodal in hepatopancreas and heart tissue at 48 h ($p < 0.01$) and bimodal in gill tissue at both 6 h and 48 h ($p < 0.01$). The expression of EhIap2 was unimodal at 24 h in hepatopancreas ($p < 0.01$), 6 h in gill tissue ($p < 0.01$), and 48 h in heart tissue ($p < 0.01$). The expression of LvIAP was also bimodal at 12 h and 72 h ($p < 0.01$) in *L. vannamei* infected with WSSV [25]. Enterohepatic circulation might explain the bimodal expression pattern in gill tissue [26]. Some crustacean IAPs such as PmIAP and LvIAP1 play key roles in the regulation of hemocyte apoptosis by inhibiting the activity of caspase [5], and circulating hemolymph has also been observed to be dramatically reduced in LvIAP2-silenced shrimp [27]. IAPs can regulate the activity of caspases to further modulate the proliferation of the cell cycle and receptor-mediated signal transduction [28]. High expression of EhIap1 and EhIap2 has been observed in immune-associated tissues [29], including hemolymph, hepatopancreas, and gill tissue, and this might be attributed to cellular metabolism and innate immunity [30].

In conclusion, two novel inhibitors of apoptosis protein genes EhIap1 and EhIap2 were identified from *E. hepuensis*; EhIap1 had two BIR domains, one UBA domain, and one RING domain, and EhIap2 had two BIR domains. EhIap1 and EhIap2 are highly homologous to the crustacean inhibitor of apoptosis protein gene (IAP). The expression of EhIap1 under azadirachtin stress was unimodal in hepatopancreas and heart tissue and had a bimodal pattern in gill tissue. The expression of EhIap2 was unimodal in immune organs. The expression of EhIap1 and EhIap2 was higher in surviving individuals than in dead individuals, and this might have contributed to the inability of some individuals to survive azadirachtin exposure [31, 32].

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

This article does not contain any studies with human participants. All animals used in this study were purchased from the market. The experimental protocols adhered to the Guidelines of Animal Care and were approved by the Animal Management and Ethics Committee of BeiBu Gulf University.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

YYL and PZ designed this project. ZYL, YZ, and MQM cultured the experimental *Eriocheir hepuensis*. QL, ZHM, ZNZ, and ZWL collected samples and extracted RNA. PZ and ZYL performed data analysis and drafted the manuscript. MQM, BYC, QLZ, and QNF participated in the sequence alignment and performed the statistical analysis. PZ and YYL revised the manuscript. All authors read and approved the final manuscript.

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References

[1] J. Wang, L. Huang, Q. Cheng, G. Lu, and C. Wang, "Complete mitochondrial genomes of three mitten crabs, Eriocheir sinensis, E. hepuensis, and E. japonica," *Mitochondrial DNA*, vol. 27, no. 2, pp. 1175-1176, 2016.

[2] N. E. Crook, R. J. Clem, and L. K. Miller, "An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif," *Journal of Virology*, vol. 67, no. 4, pp. 2168–2174, 1993.

[3] P. Obexer and M. J. Ausserlechner, "X-linked inhibitor of apoptosis protein - a critical death resistance regulator and therapeutic target for personalized cancer therapy," *Frontiers in Oncology*, vol. 4, p. 197, 2014.

[4] A. Böttger and O. Alexandrova, "Programmed cell death in Hydra," *Seminars in Cancer Biology*, vol. 17, no. 2, pp. 134–146, 2007.

[5] J.-H. Leu, Y.-C. Kuo, G.-H. Kou, and C.-F. Lo, "Molecular cloning and characterization of an inhibitor of apoptosis protein (IAP) from the tiger shrimp, *Penaeus monodon*," *Developmental and Comparative Immunology*, vol. 32, 2008.

[6] S. M. Srinivasula and J. D. Ashwell, "IAPs: what's in a Name?" *Molecular Cell*, vol. 30, no. 2, pp. 123–135, 2008.

[7] M. Arena, D. Auteri, S. Barmaz et al., "Peer review of the pesticide risk assessment of the active substance azadirachtin (Margosa extract)," *EFSA Journal. European Food Safety Authority*, vol. 16, no. 9, p. 5234, 2018.

[8] A. Mandal and S. K. Das, "Comparative efficacy of neem (azadirachta indica) and non-neem supplemented biofloc media in controlling the harmful luminescent bacteria in natural pond culture of litopenaeus vannaemei," *Aquaculture Amsterdam*, vol. 492, 2018.

[9] S. Kumar, R. P. Raman, P. K. Pandey, S. Mohanty, A. Kumar, and K. Kumar, "Effect of orally administered azadirachtin on non-specific immune parameters of goldfish *Carassius*

auratus (Linn. 1758) and resistance against Aeromonas hydrophila," *Fish and Shellfish Immunology*, vol. 34, no. 2, pp. 564–573, 2013.

[10] T. Rastogi, W. Mahmoud, and K. Kümmerer, "Human and veterinary drugs in the environment," *Encyclopedia of the Anthropocene*, vol. 5, pp. 263–268, 2018.

[11] X. Shao, D. Lai, L. Zhang, and H. Xu, "Induction of autophagy and apoptosis via PI3K/AKT/TOR pathways by azadirachtin A in Spodoptera litura cells," *Scientific Reports*, vol. 6, no. 1, Article ID 35482, 2016.

[12] Y. Liao, K. Liu, T. Ren et al., "The characterization, expression and activity analysis of three superoxide dismutases in Eriocheir hepuensis under azadirachtin stress," *Fish and Shellfish Immunology*, vol. 117, pp. 228–239, 2021.

[13] K. Liu, J. Liu, T. Ren et al., "Cloning and analysis of three glutathione S-transferases in Eriocheir hepuensis and their expression in response to azadirachtin stress," *Aquaculture Reports*, vol. 19, Article ID 100635, 2021.

[14] Q. Cai, H. Sun, Y. Peng et al., "A potent and orally active antagonist (SM-406/AT-406) of multiple inhibitor of apoptosis proteins (IAPs) in clinical development for cancer treatment," *Journal of Medicinal Chemistry*, vol. 54, no. 8, pp. 2714–2726, 2011.

[15] Z. Xing, E. M. Conway, C. Kang, and A Winoto, "Essential role of Survivin, an inhibitor of apoptosis protein, in T cell development, maturation, and homeostasis," *Journal of Experimental Medicine*, vol. 199, no. 1, pp. 69–80, 2004.

[16] V. Gesellchen, D. Kuttenkeuler, M. Steckel, N. Pelte, and M. Boutros, "An RNA interference screen ide.jpgies Inhibitor of Apoptosis Protein 2 as a regulator of innate immune signalling in Drosophila," *EMBO Reports*, vol. 6, no. 10, pp. 979–984, 2005.

[17] S. R. Fernandes, L. Barreiros, R. F. Oliveira et al., "Chemistry, bioactivities, extraction and analysis of azadirachtin: state-of-the-art," *Fitoterapia*, vol. 134, pp. 141–150, 2019.

[18] A. John and H. Raza, "Azadirachtin attenuates lipopolysaccharide-induced ROS production, DNA damage, and apoptosis by regulating JNK/akt and AMPK/mTOR-Dependent pathways in rin-5F pancreatic beta cells," *Biomedicines*, vol. 9, no. 12, p. 1943, 2021.

[19] W. Wu and X. H. Zhu, "Toxic effects on fishes and application on the parasitic diseases control by azadirachtin," *Chinese Journal of Pesticide Science*, 2003.

[20] Z. Wang, X. Cheng, Q. Meng et al., "Azadirachtin-induced apoptosis involves lysosomal membrane permeabilization and cathepsin L release in Spodoptera frugiperda Sf9 cells," *The International Journal of Biochemistry and Cell Biology*, vol. 64, pp. 126–135, 2015.

[21] F. Cossu, M. Milani, E. Mastrangelo, and D. Lecis, "Targeting the BIR domains of inhibitor of apoptosis (IAP) proteins in cancer treatment," *Computational and Structural Biotechnology Journal*, vol. 17, pp. 142–150, 2019.

[22] Q. L. Deveraux and J. C. Reed, "IAP family proteins—suppressors of apoptosis," *Genes and Development*, vol. 13, no. 3, pp. 239–252, 1999.

[23] T. Qu, L. Zhang, W. Wang et al., "Characterization of an inhibitor of apoptosis protein in *Crassostrea gigas* clarifies its role in apoptosis and immune defense," *Developmental and Comparative Immunology*, vol. 51, no. 1, pp. 74–78, 2015.

[24] C. Qu, J. Sun, Q. Xu et al., "An inhibitor of apoptosis protein (EsIAP1) from chinese mitten crab eriocheir sinensis regulates apoptosis through inhibiting the activity of esCaspase-3/7-1," *Scientific Reports*, vol. 9, no. 1, Article ID 20421, 2019.

[25] P. H. Wang, D. H. Wan, Z. H. Gu et al., "Analysis of expression, cellular localization, and function of three inhibitors of apoptosis (IAPs) from litopenaeus vannamei during WSSV infection and in regulation of antimicrobial peptide genes (AMPs)," *PLoS One*, vol. 8, Article ID 72592, 2013.

[26] L. Lin, H. Fan, B. Liao, P. Yu, and Q. Zhong, "Pharmacokinetics of sulfadiazine in european eels (*Anguilla anguilla*)," *Journal of Inspection and Quarantine*, vol. 20, no. 4, pp. 14–17, 2010.

[27] J. H. Leu, Y. C. Chen, L. L. Chen et al., "Litopenaeus vannamei inhibitor of apoptosis protein 1 (LvIAP1) is essential for shrimp survival," *Developmental and Comparative Immunology*, vol. 38, pp. 78–87, 2012.

[28] Y. L. Yang and X. M. Li, "The IAP family: endogenous caspase inhibitors with multiple biological activities," *Cell Research*, vol. 10, no. 3, pp. 169–177, 2000.

[29] Z. Y. Zhao, Z. X. Yin, S. P. Weng et al., "Profiling of differentially expressed genes in hepatopancreas of white spot syndrome virus-resistant shrimp (Litopenaeus vannamei) by suppression subtractive hybridisation," *Fish and Shellfish Immunology*, vol. 22, no. 5, pp. 520–534, 2007.

[30] M. D. Lavine and M. R. Strand, "Insect hemocytes and their role in immunity," *Insect Biochemistry and Molecular Biology*, vol. 32, no. 10, pp. 1295–1309, 2002.

[31] K. B. Choi, F. Wong, J. M. Harlan, P. M. Chaudhary, L. Hood, and A. Karsan, "Lipopolysaccharide mediates endothelial apoptosis by a FADD-dependent pathway," *Journal of Biological Chemistry*, vol. 273, no. 32, Article ID 20185, 1998.

[32] European Food Safety Authority Efsa, M. Arena, D. Auteri et al., "Peer review of the pesticide risk assessment of the active substance azadirachtin (Margosa extract)," *EFSA journal. European Food Safety Authority*, vol. 16, no. 9, p. 5234, 2018.