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Research Article
Protective Effect of Leek Extract (*Allium ampeloprasum* L.) on Catfish (*Clarias gariepinus*) Experimentally Challenged with *Aeromonas hydrophila*

[1,2]Shimaa E. Ali, [1]Waleed Soliman, [1]Iman M.K. Abumourad, [1]Mamdouh Y. Elgendy and [3]Mwansa Songe

[1]Department of Hydrobiology, Veterinary Research Division, National Research Centre (NRC), Dokki, 33 El Bohouthst, P.O. BOX 12622, Giza, Egypt
[2]WorldFish, Cairo, Egypt
[3]Ministry of Fisheries and Livestock, Department of Veterinary Services, Central Veterinary Research Institute, Lusaka, Zambia

Abstract

Background and Objective: Leek (*Allium ampeloprasum*) is one of the most commonly used herbal foods all over the world. This study was conducted to evaluate the protective effect of leek extract on catfish experimentally challenged with *Aeromonas hydrophila,* a problematic bacterial pathogen that affects various freshwater fish species. **Materials and Methods:** *Aeromonas hydrophila* was isolated and identified from catfish showing clinical signs of septicemia. The *in vitro* activity of leek extract to control the growth of *Aeromonas hydrophila* was investigated. In the *in vivo* experiment, about 240 adult catfish (*Clarias gariepinus*) were fed three different leek extract concentrations (10, 25 and 50 mg kg^{-1} body weight) for 1 month. Later on, a challenge study was conducted using an identified *A. hydrophila* strain. Morbidity and mortality were recorded throughout one week post-challenge. Furthermore, the effect of leek extract on some immune-related genes was investigated. **Results:** Under the *in vitro* testing, a significant increase (10 and 13 mm) in the inhibition zone was recorded in wells treated with 25 and 50 mg L^{-1} leak extract, respectively. A significant reduction in fish mortalities was reported in all leek extract treated groups compared to the control group which was given water. TLR1 gene expression was upregulated in fish treated with leek extract while TNFα gene expression was down-regulated. **Conclusion:** Overall, results suggested that the leek extract has immunostimulating effects that can help control bacterial infections in catfish and probably other fish species.

Key words: Leek, *Allium ampeloprasum*, *Aeromonas hydrophila*, *Clarias gariepinus*, catfish, natural immunostimulants, gene expression

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Corresponding Author: Shimaa E. Ali, Department of hydrobiology, National Research Centre, Dokki, 33El Bohouthst P.O. BOX 12622, Giza, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Aquatic diseases caused by bacterial pathogens constitute one of the major causes of economic problems in aquaculture. *Aeromonas hydrophila*, the causative agent of motile hemorrhagic septicemia, frequently causes disease in fish and other aquatic animals[1]. Antibiotics and chemotherapeutics traditionally used to keep the disease in check can lead to the emergence of drug-resistant bacteria. There are also concerns about the use of antibiotics in aquaculture due to their potential adverse effects on the aquatic environment. Recently, more interest has been directed to the development of immuno-stimulants especially those of natural origin[2].

Leek, *Allium ampeloprasum* L. belongs to the genus Allium (family Amaryllidaceae), along with onions and garlic. In addition to its affordable price, leek possesses many medicinal properties including antioxidant and antimicrobial effects[3]. In contrast to onion and garlic, little information in the literature is available regarding the immune stimulatory activity of leek against fish bacterial pathogens.

In recent years, many immune-related genes have been identified and characterized in fish and many studies have been conducted to scrutinize the expression of these genes during disease development, to understand the molecular pathogenesis. Studies conducted to investigate the role of different cytokines during bacterial infection revealed their crucial role in combating these infections[4]. Analyses of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and Toll-like receptors (TLRs) mRNA expression confirmed their involvement in inflammatory responses in zebrafish embryos and adults[5], orange-spotted grouper (*Epinephelus coioides*)[6] and rainbow trout[7].

Identification and characterization of the innate immune genes in catfish and understanding their protective mechanisms in immunity as well as their association with immune-stimulating agents/factors will help us to successfully manage disease incidents in the aquaculture industry. The present study was conducted to test the possible protective effects of leek extract against *A. hydrophila* infection in catfish, *Clarias gariepinus* and to evaluate the immunostimulatory function of leek extract. The study also investigated the potential role leek extract plays in inhibiting *A. hydrophila* growth under *in vitro* conditions.

MATERIALS AND METHODS

Study area: The study was conducted at the Department of Hydrobiology, Veterinary Division, National Research Center, Egypt from June-August, 2018. Catfish used in this study were obtained from commercial fish farms in Kafr El-Sheikh governorate.

Bacterial strain: The bacterial strain was isolated on Tryptic Soy Agar media (Merk®) from the kidney of diseased catfish showing severe clinical signs. The strain was purified and then identified biochemically using an API system (bioMérieux®)[8]. For molecular identification, DNA was extracted from the broth culture of 24 hrs growing bacteria in tryptic soy agar broth using QIAamp DNA Mini kit (Qiagen, Germany) according to manufactures instructions. The 16S ribosomal RNA gene was amplified using EmeraldAmp® GT PCR Master Mix (takara, Clontech Cat No. RR310A) and primers forward AGAGTTTGATCCTGGCTCAG and reverse GACGGGCGGTGT GTACAA were used[9]. Amplification was done by initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing of primers at 59°C for 1 min, extension at 72°C for 1 min. The final extension was at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis and size was estimated by comparison with the 1 kb Plus DNA ladder. The gene sequencing of the 16S gene was as follows: The gene-specific amplicons were excised and purified from gels using the QIAquick Gel Extraction Kit (Qiagen, Gmbh, Hilden, Germany). Further, purified PCR products were used directly for cycle sequencing reactions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Reaction products were purified and sequenced on an ABI 3500XL Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). The sequences were visualized, assembled and edited using BioEdit version 7.2.5. A standard nucleotide-nucleotide BLAST search was performed to explore the similarity to other available *Aeromonas* species sequences in GenBank.

Plant extract preparation: Leek (*Allium ampeloprasum*) leaves were collected from Egyptian local markets and air-dried. Extraction was performed according to the method described by researchers[10]. Briefly, 1.0 kg of dried leaves was, subjected to 95% alcohol. The extract was obtained by percolation and then filtered off. Using a rotator evaporator, filtrates were evaporated under reduced pressure and low temperature and a crude extract was obtained as a solid mass (powder). Three different working solutions namely 10, 25 and 50 mg (W/V), were prepared using dimethyl sulfoxide (DMSO). Obtained stock solutions were kept at 4°C and diluted before use.

***In vitro* antibacterial activity of leek extract:** The antibacterial activity of leek extract was measured using the agar well diffusion method described by Perez and others[11]. An *A. hydrophila* strain isolated and identified as described above was used. Colonies of *A. hydrophila* were transferred to sterile tubes each containing 5 mL of Tryptic Soy Broth (TSB). The turbidity of the bacterial suspensions was adjusted to an optical density equivalent to a McFarland standard tube No (1) to give a bacterial suspension of 10^8 CFU mL^{-1}. Mueller-Hinton agar plates were inoculated by streaking bacterial swabs from the previous suspension over the entire surface of the plates. The plates were then allowed to dry at room temperature and 6 mm wells were punched in each plate. Three solutions of known leek concentrations were prepared by dissolving the exact amount of leek extract into the measured volume of DMSO solvent (10, 25 and 50 mg). A total of 20 µL of each concentration was added into wells in each swabbed plate. Wells filled with the same amount of either DMSO or sterile water were included as negative controls. Plates were incubated at 28°C for 18-24 hrs, then examined for bacterial growth inhibition and zones of inhibition were measured in millimeters.

Ethics statement and *in vivo* experimental design: The experiment was carried out under the "Guidelines for the Use of Fishes in Research, 2004". Two hundred forty (240) catfish (*Clarias gariepinus*) with average body weight 100 g were divided into 4 main groups (60 fish in each), three treated with leak extract and one control group. The test groups were fed three different leek concentrations namely 10, 25 and 50 mg kg^{-1}, one concentration per group, using a pipette. The control was only given sterile water. Each group was further subdivided into 3 separate tanks consisting of 20 fish per tank. The fish were given different treatments daily for 1 month.

In vivo* antimicrobial effects of leek extract against *A. hydrophila

Bacterial strains and challenge test: A virulent bacterial strain of *A. hydrophila* was obtained as mentioned above. It was sub-cultured into TSB for 24 hrs at 28°C, washed twice with sterile Phosphate Buffer Saline (PBS), centrifuged at 3000 rpm for 10 min and then re-suspended in PBS for the needed concentration of bacterial injection (colony forming units) according to McFarland standard tubes (BioMrieux Ref 70 900 France) with a different optical density at 550 nm, using known turbidity standard. The virulence of the bacterial strain was tested by determining the LD50 by intra peritoneal (I/P) administration of various doses as previously described by Yin *et al.*[12]. The LD50 was 3×10^8 CFU mL^{-1}.

The fish were anesthetized by holding them in aquaria containing clove oil at a concentration of 50 µL L^{-1} [13]. They were then taken out of the anesthetic bath individually and intraperitoneally injected with 0.2 mL of virulent bacterial suspension after which they were transferred to their holding tanks.

The fish were observed for their response against injected *A. hydrophila* and the mortality pattern was recorded for 1 week after injection. Dead and scarified fish were examined for gross lesions and postmortem changes in the internal organs. Bacteriological swabs were taken from kidneys of scarified fish showing external or internal lesions and sub-cultured onto TSA for re-isolation and further identification of *A. hydrophila.* RPS (relative percent survival) of challenged fish in different groups during 1 week was calculated according to Ngugi *et al.*[14] by this Eq.:

$$\text{RPS} = \frac{\text{Number of surviving fish after challenge}}{\text{Total number of fish injected with bacteria}} \times 100$$

Molecular evaluation of nonspecific immunity using real-time PCR for TNF-α and TLR-1 gene expression

RNA extraction and cDNA synthesis: Three days after bacterial injection, livers were dissected from the infected and control fish and frozen immediately in RNA later and stored at -80°C. RNA was further extracted using Gene Jet RNA Purification Kit and the concentrations were measured spectrophotometrically.

About 2 µg RNA was reverse transcribed with Revert Aid First Strand cDNA Synthesis Kit™ (purchased from Fermentas life science Co., Invitrogen Corporation), using hexanucleotides and used as templates for real-time PCR. The reaction was performed using a Bio-Rad thermo-cycler machine; at 45°C for 60 min after which the reaction was stopped by rising the temperature to 70°C.

Primer designing: Primers for target genes and 18s ribosomal RNA were designed with the aid of the Primer5 program (Table 1) and was purchased from Invitrogen Corporation (Van Allen Way, Carlsbad, Canada).

Quantitative real-time PCR: The cDNA was PCR amplified using corresponding primers for TNFα, TLR-1 and 18S rRNA was used as a reference housekeeping gene (GenBank accession No. AJ876383). PCR Applied Biosystems was employed with the use of SYBR Green PCR Master Mix

Table 1: Sequences of TNF-α and 18s *rRNA* genes

Gene	Sequence	Strand
18sRNA	5'TTAGTTGGTGGAGCGATTTG3'	F
	5'GGACATCTAAGGGCATCACA3'	R
TNF-α	5'TCTCAGGTCAATACAACCCGC-3'	F
	5'GAGGCCTTTGCGGAAAATCTTG-3'	R
TLR-1	5' AGCGAAAGAAATGCCAGCTG 3'	F
	5' TGACGTCTCGTTCGTGCTGA -3'	R

(Applied Biosystems) and gene-specific primers where specimens analysis were done in a final volume of 12 µL in MicroAmp® Optical 96-well reaction plates (Applied Biosystems, Foster City, CA) using Applied Biosystems (ABI) 7500 Real-Time PCR System (The qPCR mixture consisted of 1 µL of cDNA (equivalent to 10 ng of RNA), 1 µL of 0.5 mM gene-specific forward primer, 1 µL of 0.5 mM gene-specific reverse primer, 6 µL of 2×SYBR Green Super Mix and 3 µL of DEPC-treated water. Q PCR was performed in triplicate for each cDNA sample. The qPCR thermal cycling parameters were 50°C for 2 min, 95°C for 2 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The PCR products were also gel-excised, purified and sequenced to confirm that they match target genes sequences.

Data analysis: The relative transcriptional levels of different genes were determined by subtracting the Cycle Threshold (Ct) of the sample by that of the 18S ribosomal RNA, the calibrator, using the formula:

$$\Delta Ct = Ct\ (sample) - Ct\ (calibrator)$$

The relative expression level of a specific gene in the immunized fish was compared to that of non-immunized fish to obtain $2\Delta\Delta Ct$, where, $\Delta\Delta Ct = Ct\ (control) - \Delta Ct$[15]. Statistical analyses for the mRNA transcription levels were performed with the aid of the SPSS.16 statistical package (SPSS Inc., Microsoft Co., Redmond, USA). Two-way analysis of variance (ANOVA) was used to test the effects of experimental feed for all parameters while student's t-test was used to test differences among individual means and the control. The difference was recorded as significant when $p<0.05$.

RESULTS

Bacterial strain: The biochemical and molecular identification revealed that the isolated bacteria had the highest sequence similarity to *Aeromonas hydrophila* giving 99% identity. The sequence with a length of 1400 bp. was obtained and subsequently uploaded to NCBI (GenBank accession number, MT524304).

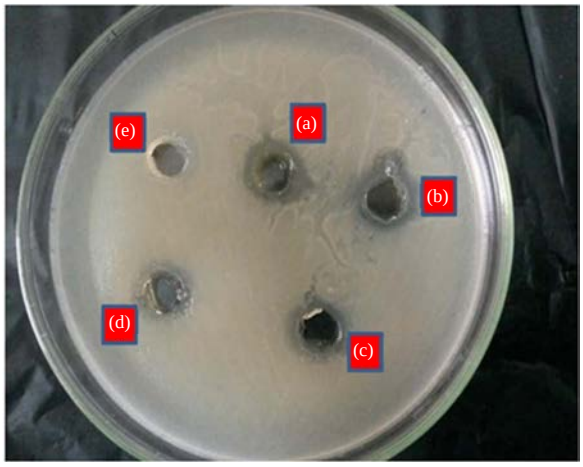

Fig. 1(a-e): Antibacterial activity of leek extract on *Aeromonas hydrophila* growth compared to water and DMSO, (a) 13 mm (50), (b) 10 mm (25), (c) 7 mm (10), (d) 7 mm (DMSO) and (e) Zone of inhibition = 0 mm (DW)

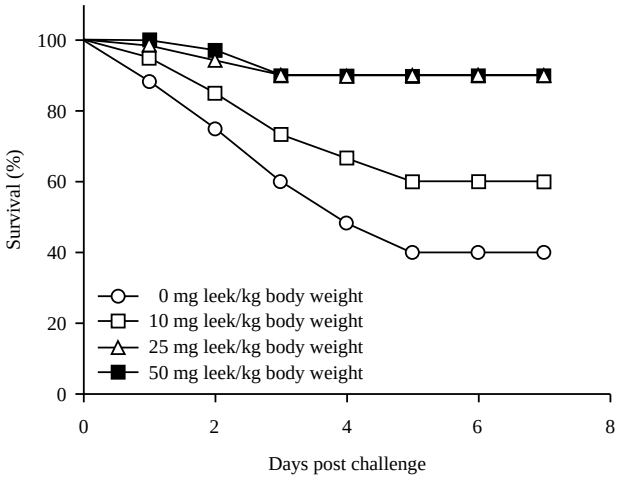

Fig. 2: Post challenge survival curve of leek treated *Clarias gariepinus* catfish compared to control
The survival rate reached 40% (24 fish) in the control group compared 60% survival (36 fish) in the fish group fed 10 mg kg^{-1} leek extract. Survival rate reached 90% (54 fish) in both fish groups fed 25 and 50 mg kg^{-1} leek extract

***In vitro* antibacterial activity of leek extract:** Concentration-dependent increase in the inhibition zone was recorded in wells treated with 25 and 50 mg L^{-1} (10 and 13 mm, respectively) (Fig. 1a, b). However, weak antibacterial activity against *A. hydrophila* was observed in leek extract-treated wells at a concentration of 10 mg and DMSO (7 mm) (Fig. 1c, d). No inhibition was observed in the negative control (water treated well, Fig. 1e).

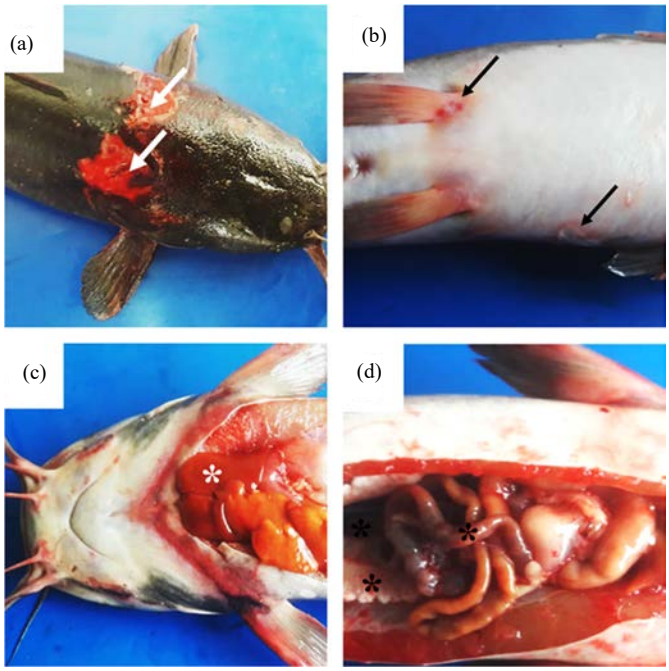

Fig. 3(a-d): Diffuse hemorrhage and ulceration affecting skin and muscle of the head region of catfish experimentally infected by *Aeromonas hydrophila* in the non-treated control group that received 0% leek

(a) Compared to mild tiny hemorrhagic lesion in catfish which received the lowest leek concentration (10% mg kg^{-1}) (b), (C) and (d) are showing the postmortem changes induced by *A. hydrophila* in the non-treated control group, (c) Is showing congested liver, swelling of the gall bladder, spleen and kidney (white asterisk) while and (d) is showing the enlargement of gonads, liver and kidney with hemorrhagic intestine (black asterisk)

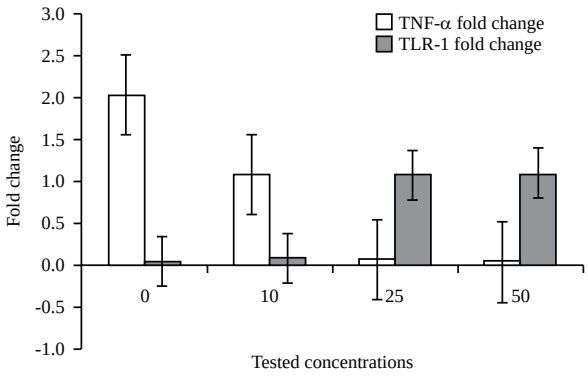

Fig. 4: Fold changes of TNF-a and TLR-1 with different leek concentrations

Challenge experiment: The survival rate reached 40% in the control group experimentally infected with *A. hydrophila* (24 survived fish). Compared to that, a slight increase in the survival level was recorded in the fish group fed 10 mg kg^{-1} leek extract before the challenge test (36 survived fish representing 60%). The highest survival rate (54 fish/group) was detected in the fish group fed 25 and 50 mg kg^{-1} leek extract with both groups showing 90% survivability (Fig. 2).

The observed clinical signs in the non-treated control group (0% leek) included hemorrhagic ulcerative lesions on the skin and fins (Fig. 3a). For the Post Mortem (PM) examination, enlargement of gonads, kidney and liver was observed with intestinal haemorrhage (Fig. 3b). The gall bladder was also distended with swollen spleen and kidney (Fig. 3c). In contrast, few, tiny hemorrhagic lesions were observed on the skin of catfish fed on leek at a concentration of 10% only (Fig. 3d). At PM examination, no obvious changes were detected.

Real-time quantitative PCR for TNF-α and TLR-1 gene expression: TNF-α gene expression was down-regulated in fish groups treated with different concentrations of leek and then challenged with pathogenic *A. hydrophila*. In contrast, the expression of the TLR-1 gene was up-regulated in the liver with the increase in leek concentration in the fish treated groups than the control with fold change 15-27 fold (Fig. 4).

DISCUSSION

Findings from the present study have given a strong indication that the leek extract has immuno-stimulating effects that can be very useful in the control of bacterial

infections in catfish. This was evidenced by a number of results such as the significant reduction in fish mortalities observed in all leek extract-treated groups (10-40%) compared to the negative control group where the mortality rate reached 60%.

The use of natural immuno-modulators for enhancing host defense responses is one of the most promising alternatives to classical medical treatment. In recent decades polysaccharides, including pectic substances isolated from plant sources, have attracted a great deal of attention because of their broad-spectrum therapeutic properties and relatively low toxicity. Onion and onion-like extracts have been found to improve immune function[16] suggested that the pectic polysaccharides from leek have immune-enhancing effects[17] isolated saponin from the leek bulb and found that it has an adjuvant activity on the cellular immune response. In fact, since prehistoric times, herbs were the basis for nearly all medicinal therapy until synthetic drugs were developed in the 19th century[18].

In the current study, *A. hydrophila* was isolated and identified biochemically and molecularly from catfish showing clear signs of septicemia. Although *A. hydrophila* is considered an opportunistic pathogen, it has been shown that some virulent strains can contribute to disease occurrence in catfish[19]. Several studies have shown that various plant extracts can exert antimicrobial activities against some fish pathogens[18]. Such natural products could be a good alternative to antibiotics especially for bacteria with antibiotic resistance profiles. In this study, we have found that leek extract exhibits antimicrobial activity on the *in vitro* growth of *A. hydrophila* in a dose-dependent pattern. Several bioassays such as well diffusion, disk-diffusion and broth or agar dilution are commonly used to evaluate the antimicrobial activity of antimicrobial agents from various sources. To investigate the *in vitro* antibacterial activity of leek extract, the current study opted for the agar well diffusion method, a procedure that is widely used to evaluate the antimicrobial activity of plants or microbial extracts[20,21]. In this method, a volume of the microbial inoculum is spread over the entire agar surface and then a quantity of antimicrobial agent or extract at the desired concentration is introduced into a well with a diameter of 6-8 mm, punched aseptically with a sterile cork borer or a tip. Researchers with an interest in demonstrating the efficacy of the test substance have used an antimicrobial agent well known to effectively treat infections caused by the bacterial pathogen in question as a positive control[22]. Follow-up efficacy and effectiveness studies on the leek extract are imperative as the use of such medicinal plants is eco-friendly and cost-effective. This makes them desirable for utilization in aquaculture health management and for addressing the issues of growing antibacterial resistance, which is of global concern.

We have found that catfish which received leek treatment at different concentrations expressed higher survival rate following experimental challenge with virulent *A. hydrophila* strain isolated from catfish during a natural outbreak. This could be attributed to the enhancement of the non-specific immune responses of fish which considered being a very important tool in controlling pathogenic infections in fishes[23]. The mortality rate reached 60% in the control group compared to 40% mortality in the fish group fed 10 mg kg^{-1} leek extract before the challenge test. The least mortality was detected in the fish group fed 25 and 50 mg kg^{-1} leek extract with both groups showing only 10% mortality. The observed clinical signs and post-mortem findings during the challenge test in this study were similar to those reported by Bannai[24].

In mammals, TNF-α which is an inflammatory mediator secreted by several inflammatory cell types is secreted directly by hepatocytes and Kupffer cells in the liver. Pathways activated by TNF-α could lead to hepatocyte apoptosis[25] and inhibition of TNF-α production can be effective in reducing liver damage[26]. Similar to mammals TNF-α in fish is believed to play a role in apoptosis and disease containment especially during bacterial infections[27]. Our real-time qPCR data demonstrated significant down-regulation of TNF-α gene expression (Fig. 4) in fish treated with leek and then infected with *A. hydrophila*. Despite that, higher survival was reported in leek extract fed fish. In some cases, inflammatory responses can be the main drivers of pathology and their induction can be detrimental. It is possible that TNF-α mediated response does not contribute to the control of the infection during *A. hydrophila* infection in fish but rather promote disease. However, this requires further studies.

Toll-like receptors (TLRs) are the first Pattern recognition receptors (PRRs) to be identified[28]. They play a crucial role in the activation of innate immunity by recognizing pathogen-associated molecules[29]. In this study, a dose-dependent up-regulation of Toll-like receptor gene TLR-1 was observed in the liver for almost all concentrations in fish fed with leek extract and then challenged with the *A. hydrophila* bacteria. A previous study on the expression of immune genes in catfish[30] also demonstrated significant up-regulation (22-27 folds) of genes TLR-1 and TLR-9 in the skin of immunized fish day one post-vaccination. A long period of stable up-regulation in the skin from 4 hrs to day 10 post vaccinations was reported. The activation of the TLRs is essential for the inflammatory response as TLRs can activate macrophages

to produce inflammatory cytokines and chemokine and can also enhance the phagocytic activity of macrophages[30]. Since less mortality was observed in fish expressing a high amount of TLR, our data, together with the above-mentioned studies, suggest that leek extract can be used to stimulate immune responses that aid in combating *A. hydrophila* and possibly other bacterial infections. This also points to the importance of leek as a feed/feed additive in raising fish immunity in aquaculture.

CONCLUSION

Mortality data as well as TNF-α and TLR-1 gene expression results obtained in fish treated orally with leek and then infected experimentally with *A. hydrophila* suggest that leek has a role in the improvement of fish immunity against *A. hydrophila* and possibly other bacterial infections. Leek extract dissolved in DMSO can inhibit the growth of *A. hydrophila* under *in vitro* conditions as observed in this study. There is a need for further research to identify the active compound responsible for such anti-*A. hydropila* activity and the exact mechanism of inhibition.

SIGNIFICANCE STATEMENT

Using catfish (*C. gariepinus*), the present study highlights the protective role of natural plants such as leek against *A. hydropila* which can be associated with mass mortalities in aquaculture. These findings will encourage researchers to pay more attention to the use of natural products to replace or reduce the use of antibiotics to control fish pathogens that pose a serious threat to the aquaculture industry.

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