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RESEARCH

The therapeutic role of *Azadirachta indica* leaves ethanolic extract against detrimental effects of *Aeromonas veronii* infection in Nile tilapia, *Oreochromis niloticus*

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Abstract Bacterial pathogens cause high fish mortalities and in turn economic losses in fish farms. Innovative strategies should be applied to control bacterial infections instead of antibiotics to avoid the resistance problem. Consequently, the present investigation studied the curative potential of *Azadirachta indica* leave ethanolic extract (AILEE) on *Aeromonas veronii* infection in *Oreochromis niloticus.* A preliminary trial was assessed to evaluate the curative dose of AILEE which was found to be 2.5 mg/L. One hundred and sixty fish were divided into equal four groups in four replications, where group 1 and group 2 were non-challenged and treated with 0- and 2.5-mg/L AILEE, respectively. Group 3 and group 4 were challenged with *A. veronii* and treated with 0- and 2.5-mg/L AILEE, respectively for 10 days. *A. veronii* infection produced severe clinical manifestations and a high mortality rate in the infected fish. Furthermore, the infected fish exhibited a significant rise in the hepatorenal indices (aspartate aminotransferase, alanine aminotransferase, and creatinine), the oxidant biomarker (malondialdehyde), and the stress indicators (glucose and cortisol). A significant reduction in the protein profile and antioxidant/immune parameters (catalase, immunoglobulin M, lysozyme, nitric oxide, and phagocytic activity) was observed in the infected fish. Water application of the infected group to 2.5-mg/L AILEE notably ameliorated the hepatorenal indices, the oxidant biomarker, and the stress indicators. Furthermore, AILEE improved the antioxidant/immune indices. Water application of 2.5-mg/L AILEE could be useful against *A. veronii* infection in *O. niloticus* culture.

Keywords Antibacterial activity · *Aeromonas veronii* · *Azadirachta indica* · Immune/antioxidant

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Introduction

Aquaculture is one of the rapidly expanding agribusiness sectors, and in the last decade, it has contributed in economic development globally, due to the

increasing need for animal protein for human consumption and the continuous supply of raw materials for food and pharmaceutical industry (Food and Agriculture Organization (FAO) of the United Nations 2022). The Nile tilapia (*Oreochromis niloticus*) is an important freshwater species in tropical and subtropical areas, which is marked by a high growth rate and consumer acceptance (Ibrahim et al. 2021, 2019a). Extensive aquaculture practices were necessary to meet the world's growing food demand, but this led to the emergence of illnesses (Araujo et al. 2022).

The main cause of the massive annual losses in the aquaculture industry, which are evaluated to be worth billions of US dollars, is bacterial infections (Abdelrahman et al. 2023). Bacterial diseases are the biggest obstacle to the growth of aquaculture (Rajme-Manzur et al. 2021). Tilapia is vulnerable to several bacterial diseases in the stressful environment of intensive farming (Medina et al. 2020; Mugwanya et al. 2021). Motile aeromonads are common disease agents in fish farms (Haenen et al. 2023). Because of Aeromonas spp., *O. niloticus* culture experiences high economic losses and severe mortality (Raj et al. 2019). Within the Aeromonas genus, *Aeromonas veronii* is a noteworthy species that causes significant mortality in numerous fish species, including *O. niloticus* (Smyrli et al. 2022). *A. veronii* is gram-negative pathogen that has reportedly threatened human health as well as caused significant economic losses for the fish farming industry (Sun et al. 2016). Epizootic ulcerative syndrome (EUS), motile *Aeromonas septicemia*, hemorrhagic septicemia of fish, and other illnesses are linked to *A. veronii* (Abdel Rahman et al. 2022). Ocular disorders, bilateral exophthalmia, hemorrhagic septicemia, and ulcers were noted in *O. niloticus* infected with *A. veronii* (Bispo dos Santos et al. 2023).

Excessive and repeated use of antibiotics for treating bacterial illness in aquaculture lead to their accumulation in fish tissues and promotes the formation of antibiotic-resistance problem (Binh et al. 2018). Antibiotic-resistant bacterial strains arise and spread mostly as a result of the overuse of antibiotics. These infections are very dangerous to aquatic life and human health (Serwecińska 2020).

Finding eco-friendly and effective alternatives for treating bacterial illness is a must for reducing antibiotic use in aquaculture (Abdel Rahman et al. 2023a, b; Ibrahim et al. 2024). Numerous biological functions, including antioxidant, anti-inflammatory, and antibacterial ones, have been found in medicinal plants (Ahmed et al. 2022; Ibrahim et al. 2019b). Medicinal plant antimicrobial activity is a novel possibility for addressing the serious risks posed by rising evidence of antibiotic resistance. Consequently, the identification and isolation of novel bioactive compounds from medicinal plants—which have not yet received enough research—is urgently needed. These substances' great diversity has demonstrated their therapeutic potential as antimicrobials and as modulators of antimicrobial resistance (Odongo et al. 2023; Vaou et al. 2021).

Among the recently used medicinal plants, *Azadirachta indica* (*A. indica*), known as neem, is a member of the *Meliaceae* (mahogany) family (Haque et al. 2016). "Village pharmacy", "Tree of the 21st century", and "A tree for tackling world problems" are some of the names given to *A. indica* (Islas et al. 2020; Kaur et al. 2020). *A. indica* is a plant with antibacterial, anti-inflammatory, antipyretic, and immunocontraceptive effects (Dhillon et al. 2024; Sarkar et al. 2021; Wylie and Merrell 2022). *A. indica* leave extract, which has polyphenols that adhere to oral surfaces, can have long-lasting antibacterial and synergistic antioxidant effects when combined with bacteria (Heyman et al. 2017). Dietary *A. indica* leave extract boosted the growth and immune responses of common carp (*Cyprinus carpio* Linnaeus) (Kaur et al. 2020) and improved the growth and health of Rainbow trout (*Oncorhynchus mykiss*) (Abidin et al. 2022). The dietary *A. indica* leave ethanolic extract (AILEE) boosted the hematological variables and increased disease resistance to co-infections of *Streptococcus agalactiae* and *Aeromonas jandaei* (Abarike et al. 2022a, b). AILEE exhibited in vitro antibacterial activities against *Staphylococcus aureus* and *Aeromonas hyprophila* in Singhi (*Heteropneustes fossilis*) and Mrigal (*Cirrhinus mrigala*) (Devi et al. 2019). In addition, AILEE had in vitro antibacterial activities against *A. jandaei* in Nile tilapia (Abarike et al. 2022a, b).

The hepato-renal indices (aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine (Creat)) are indicators for the liver and kidney functions in fish (Abdel-Daim et al. 2020). Malondialdehyde (MDA) is the end product of lipid peroxidation and an indicator for oxidative stress while, catalase (CAT) is an antioxidant enzyme (Köprücü et al. 2023). *A. indica* leave was previously reported to have a hepato-renal protective effect and helped to restore the liver and kidney

functions, as well as increased the activity of antioxidant enzyme and reduced MDA level as a result of its biological constituents in Nile tilapia (Ibrahim et al. 2022c). Total proteins (TP), albumin (ALB), globulin (GLB), lysozyme (LYS), nitric oxide (NO), and phagocytic activity (PA) are considered non-specific immune indicators (Ahmed et al. 2021; Zheng et al. 2020). Immunoglobulin M (IgM) is the predominant immunoglobulin and an adaptive immune indicator in fish (Bai et al. 2022). Previous study had demonstrated that *A. indica* leave extract boosted the non-specific and specific immune responses in striped catfish (*Pangasianodon hypophthalmus*) (Nhu et al. 2019). We established this work based on the serious effects of *A. veronii* on the health and productivity of Nile tilapia, as well as the hazards related to the use of antibiotics in aquaculture on fish health and, ultimately, consumer welfare. This work was intended to estimate the probable treatment effect of AILEE as an eco-friendly alternative against *A. veronii*–caused hepato-renal disorders and antioxidant-immune dysfunction in Nile tilapia.

Materials and methods

Preparation of plant extract

Fresh *Azadirachta indica* leaves (Desert Research Center, Cairo, Egypt) were air dried in a dark room, then ground into uniform powder using a milling machine (Moulinex mixer 716, France) to make extract using ethanol 70%. Using a rotary evaporator, the produced extract was concentrated and kept for further use (Ibrahim et al. 2023).

Antioxidant activity evaluation (DPPH radical scavenging activity assay)

The antioxidant capacity of AILEE with papain was evaluated using the DPPH radical scavenging activity reported by Göçer and Gülçin (2011), with minor adjustments. Four milliliters of 0.15 mM DPPH were combined with one milliliter of AILEE. After that, a mixer was used to vigorously stir the liquid. At room temperature, the reaction mixture was incubated for 30 min in complete darkness. Using a spectrophotometer, the absorbance of the resultant solution at 517 nm was assessed. The radical-scavenging capacity of the sample was evaluated as a function of a decrease in DPPH radical absorbance using the following equation:

$$\text{Radical scavenging activity (\%)} = [(\text{A control} - \text{A sample})/\text{A control}] \times 100$$

With A= absorbance at 517 nm.

Gas chromatography-mass spectrometry analysis (GC-MS)

The GC-MS system (Agilent Technologies) was supplied with a gas chromatograph (7890B), mass spectrometer detector (5977A), and HP-5MS column at the National Research Centre, Cairo, Egypt. Hydrogen as a carrier gas was used at a flow rate of 1.0 ml/min and kept for 20 min. The injector and detector were held at 250 °C. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 30–700 and solvent delay of 9 min. The mass temperature was 230°C and Quad 150 °C. By contrasting the spectrum fragmentation pattern with those found in Wiley and NIST Mass Spectral Library data, the different compounds were identified.

Determination of the phytochemical constituents of AILEE

To assess the phytochemical elements of AILEE (phenolics, terpenoids, saponins, flavonoids, alkaloids, tannins, steroids, resins, and glycosides) qualitatively, a standard methodology (Trease and Evans 1989) was applied.

Isolation of the bacterial strain

An *A. veronii* strain (131TF-ID) was identified from a sick *O. niloticus* at the Aquatic Animal Medicine Department, Faculty of Veterinary Medicine, Zagazig University, Egypt (Reda et al. 2022). Utilizing the VITEK® 2 system (BioM' erieux Inc., NC, USA) to identify the isolate and confirm its pathogenicity to *O. niloticus*. *A. veronii* was cultivated for 24 h on tryptic soy agar (Himedia®) at 27 °C, and one colony was chosen to incubate for a further 24 h in tryptic soy broth (Himedia®) at 27 °C. The *A. veronii*–cultured broth was centrifuged at 3000 ×*g* for 10 min at 4 °C to get the pellet, which was then mixed in a sterile phosphate–buffered saline solution.

Disc diffusion assay

The disc diffusion technique was used to assess the inhibition zone during the antibacterial screening of AILEE (Liu et al. 2016). Sterilized paper discs were soaked in 80% and 100% from AILEE; the discs were left to dry. Then, one colony from the 24-h-old culture of *A. veronii* was picked up and mixed with 1 ml of the sterilized saline solution and consequently poured on the nutrient agar plates. The dried discs were then put on the agar plates and afterward incubated for 24 h at 37 °C. Finally, the results were recorded and the inhibition zone (mm) was measured.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

AILEE was tested for MIC assay in triplicate (Swain et al. 2014). Serial dilutions (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml) from AILEE were prepared. The cultures containing 10^6 CFU/mL of the bacterial isolate were added, and the 96-well plate was incubated as before. The MIC at which there was no evidence of bacterial growth on the plate. By sub-culturing from each well onto a nutrient agar plate, MBC was achieved. For that test strain, the plate with the least concentration of the extract that failed to exhibit growth on subculture was regarded as MBC (Abdel Rahman et al. 2023a, b).

Fish and stocking conditions

Healthy 360 *O. niloticus* (35 ± 2.10 g) was provided by the Faculty of Veterinary Medicine's Fish Research Unit, Zagazig University of Egypt. The fish were examined clinically upon arrival to be sure that they were healthy according to CCoA (2005). The fish were reared in 60-L glass aquariums (40 × 30 × 80 cm) with dechlorinated tap water at a capacity of 10 fish/aquarium and adapted for 15 days before the beginning of the experiment. The laboratory's water criteria were dissolved oxygen (6.22 ± 0.90 mg/L), pH (6.4 ± 0.4), temperature (27 ± 2.1 °C), and ammonia (0.030 ± 0.01 mg/L) with adjusted day to night time (12 h dark:12 h light). The fish were fed three times daily on a basal diet (3% of their biomass). During the adaptation, preliminary, and experimental periods, the water was completely changed 3 times per week, and the excretory materials were siphoned out daily.

Determination of the therapeutic dose of AILEE

One hundred fish were exposed to ten grading concentrations of AILEE (0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 mg/L) to determine the proper concentration for the treatment study (Table 1). Regular daily recordation of the clinical signs and deaths was performed. The safe concentration of AILEE was 2.5 mg/L.

Treatment study

According to a prior study (Ibrahim et al. 2022a), the lethal dosage 50 (LD_{50}) of *A. veronii* was determined. Fish ($n = 100$) were divided into five groups with two replicates (10 fish/replicate). Before the experimental infection, fish were fasted for 24 h. 0.2 mL of *A. veronii* bacterial suspension (10^5–10^8 CFU/fish) was intraperitoneally (IP) injected into the first to the fourth group, while the fifth group was IP injected with 0.2 mL of sterile saline. The fish mortalities were recorded for 4 days after the injection. According to the results of the Probit Analysis Program (version 1.5), the LD_{50} was computed to be 2×10^7 CFU/mL. A sub-lethal dosage of 0.5×10^7 was adjusted using a McFarland standard tube and applied in the treatment study.

Table 1 Effect of water exposure to different AILEE levels on mortality and clinical signs of *O. niloticus* for 10 days

Conc. (mg/L)	Mortality ($N = 10$)	Clinical observations		
		Loss of escape reflex	Swimming abnormalities	Skin lesions
0.0	0/10	-	-	-
1	0/10	-	-	-
1.5	0/10	-	-	-
2	0/10	-	-	-
2.5	0/10	-	-	-
3	1/10	-	-	-
3.5	1/10	-	-	-
4	1/10	-	-	-
4.5	2/10	+	+	+
5	2/10	+ + +	+ +	+

$^{-}$No abnormal observations, $^{+}$mild abnormal observations, $^{++}$moderate abnormal observations, $^{+++}$sever abnormal observations

For the treatment study, 160 fish were haphazardly assigned into four equal groups (40 fish/group, 10 fish/replicate) for 10 days. Group 1 (control) and group 2 (AILEE) were treated with 0 and 2.5 mg/L of AILEE as water exposure, respectively, without *A. veronii* challenge. Group 3 (*A. veronii*) and group 4 (*A. veronii* + AILEE) were challenged with 0.2 mL of *A. veronii* (0.5×10^7 CFU) and treated with 0- and 25-mg/L AILEE, respectively. All clinical signs and mortalities were recorded during the experimental duration.

Sampling

At the end of the study (10 days), twelve fish from each group were chosen at random to collect blood samples from the caudal vessels. The fish were fasted for 24 h before the sample collection. According to a prior study (Lugo et al. 2008), the fish were given a 100-mg/L benzocaine solution for sedation. To separate the serum, the first set of blood samples was drawn using a 1-mL plastic syringe without anticoagulant and kept at room temperature (23 °C) for 4 h. Afterward, samples were centrifuged at 3000 ×*g* for 10 min to separate the serum, and they were then kept at 20 °C until biochemical and immunological tests. A 1-mL heparinized syringe was used to collect the second batch for the PA assay. Additionally, samples ($n = 12$/group) of the liver tissue of the dead fish were gathered to determine hepatic oxidant/antioxidant tests.

Hepatorenal and antioxidant parameter

A spectrophotometric approach was followed for measuring the serum activities of AST (catalog no. MBS1601734) and ALT (catalog no. MBS038444) (MyBioSource Co., Egypt) using a spectrophotometer (Lambda EZ201; Perkin Elmer). Moreover, the Creat level was estimated according to Fossati et al. (1983). The hepatic levels of MDA were spectrophotometrically measured in the hepatic homogenate using a specific kit (MDA, catalog No. MD 25 29); similarly, CAT was measured following Aebi (1984).

Biochemical indices

Serum proteins' electrophoretic distribution, including TP, ALB, and GLB, was measured as previously mentioned (Badawi 1971). Serum glucose (GLU) level was assessed as previously described (Nikkila 1962). Using an ELISA kit (MBS704055) from MyBioSource Co. (Cairo, Egypt), plasma cortisol (CORT) levels were measured.

Immunological indices

According to the manufacturer's instructions, we used the kits from Cusabio Co. (Houston, TX, USA) to measure IgM (catalog no. CSB-E12045Fh). Following the protocol (Ghareghanipoora et al. 2014), with minor changes, we assessed serum LYS activity using the lysis of *Micrococcus lysodeikticus* (Sigma Co., MO, USA). We combined the serum with the *M. lysodeikticus* solution (0.2 mg/mL in 0.05 M PBS, pH 6.2) at 25 °C for 5 min. We employed a 5010 photometer from the BM Co. in Berlin, Germany, to measure the optical density at 540 nm for 5 min at 1-min intervals. With the use of various dilutions of lyophilized chicken egg-white LYS (Sigma Co., MO, USA). Following Bryan and Grisham (2007) methodology, NO was evaluated. Following the Cai et al. (2004) methodology, the PA% of leukocytes was evaluated.

Statistical analysis

Shapiro–Wilk's test was utilized to determine the normality of the data. A two-way analysis of variance (ANOVA) was performed on the results of the biochemical, immunological, and oxidant/antioxidant indices using SPSS version 18 (SPSS, Chicago, IL, USA). With a significance threshold of 0.05, Duncan's multiple range tests were employed to find differences between means ($N = 12$ per group).

Results

DPPH activity and bioactive compounds of AILEE determined by GC–MS

The DPPH activity of AILEE was 77%. The result of GC-MS of AILEE is shown in Table 2. The GC-MS detected 20 compounds in AILEE. The major compounds are D-(-)-fructofuranose, pentakis(trimethylsilyl) ether (isomer 2) (25.64%), D-(-)-fructofuranose, pentakis(trimethylsilyl) ether (isomer 1) (25.04%),

D-fructose, 5TMS derivative (10.55%), D-(+)-talofuranose, pentakis(trimethylsilyl) ether (isomer 1) (6.23%), D-psicofuranose, pentakis(trimethylsilyl) ether (isomer 1) (4.26%), 3,8-dioxa-2,9-disiladecane, 2,2,9,9-tetramethyl-5,6-bis[(trimethylsilyl)oxy] (3.24%), malic acid, 3TBDMS derivative (3.24%), palmitic Acid, TMS derivative (2.74%), α-linolenic acid, TMS derivative (2.55%), arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)- (2.18%), D-lyxose, 4TMS derivative (2.09%), D-xylopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)- (2.07%), 1,3-dihydroxyacetone dimer, 4TMS derivative (2.02%), beta-D-galactofuranoside, and ethyl 2,3,5,6-tetrakis-O-(trimethylsilyl)- (1.99%).

Phytochemical composition of the plant extract

The active phytochemical compounds of AILEE are presented in Table 3. AILEE had an abundant amount of terpenoids, saponins, and alkaloids followed by phenolics, tannins, glycosides, and flavonoids with the absence of steroids and resins.

In vitro antibacterial activity

Figure 1 demonstrated that AILEE had an inhibitory zone of 20 mm at 80% concentration and 23 mm at 100% concentration when used against *A. veronii.* The MIC of AILEE against *A. veronii* was 40 µg/mL, while, the MBC was 50 µg/mL.

Clinical signs, behavior, and mortalities

Non-infected fish (control and AILEE groups) exhibited normal clinical signs with 0% mortalities (Fig. 2), in addition to normal swimming behavior, and responded well to different body reflexes (escape and tail reflexes). Infected fish treated with 0-mg/L AILEE (*A. veronii*) suffered severe clinical manifestation in the form of fin rot, skin hemorrhage, skin ulcer, and scale loss, as well as high mortality rate (35%), additionally slow swimming activity with low response to different body reflexes. On the other side, infected fish treated with 2.5-mg/L AILEE (*A. veronii*+ AILEE) retrieved the previously mentioned signs except for slight fin rot and dark body color

Table 2 Bioactive compounds in AILEE determined by GC–MS

Name	Formula	RT (min)	Peak area %
D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 2)	C21H52O6Si5	26.742	25.64
D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 1)	C21H52O6Si5	26.634	25.04
D-Fructose, 5TMS derivative	C21H52O6Si5	26.542	10.55
D-(+)-Talofuranose, pentakis(trimethylsilyl) ether (isomer 1)	C21H52O6Si5	27.006	6.23
D-Psicofuranose, pentakis(trimethylsilyl) ether (isomer 1)	C21H52O6Si5	26.445	4.26
3,8-dioxa-2,9-disiladecane, 2,2,9,9-tetramethyl-5,6-bis[(trimethylsilyl)oxy]	C16H42O4Si4	26.817	3.34
Malic acid, 3TBDMS derivative	C22H48O5Si3	27.166	3.24
Palmitic acid, TMS derivative	C19H40O2Si	28.625	2.74
α-Linolenic acid, TMS derivative	C21H38O2Si	30.158	2.55
Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-	C17H42O5Si4	26.29	2.18
D-Lyxose, 4TMS derivative	C17H42O5Si4	27.429	2.09
D-Xylopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)-	C17H42O5Si4	27.572	2.07
1,3-Dihydroxyacetone dimer, 4TMS derivative	C18H44O6Si4	25.924	2.02
beta-D-Galactofuranoside, ethyl 2,3,5,6-tetrakis-O-(trimethylsilyl)-	C20H48O6Si4	26.187	1.99
Phosphoric acid, dioctadecyl ester	C36H75O4P	29.826	1.93
D-Xylose, 4TMS derivative	C17H42O5Si4	28.327	1.29
D-Ribose, 4TMS derivative	C17H42O5Si4	28.161	1.15
Glycerol, 3TMS derivative	C12H32O3Si3	19.327	0.87
Prostaglandin F-2ALPHA-TETRATMS	C32H66O5Si4	25.998	0.55
(R)-1-Benzylthio-3-(t-butyldiphenylsiloxy)-2-methoxypropane	C27H34O2SSi	38.495	0.27

Table 3 Phytochemical compounds in AILEE

Constituents	AILEE
Phenolics	+ +
Terpenoids	+ + +
Saponins	+ + +
Flavonoid	+
Alkaloid	+ + +
Tannins	+ +
Steroid	-
Resins	-
glycosides	+ +

$^{-}$Not detected; $^{+}$detected in low amount; $^{++}$detected in moderate amount; $^{+++}$detected in high amount

with a 10% mortality rate. (*A. veronii*+ AILEE) the group had better swimming activity and response to the body reflexes compared to the infected one but lower than the non-infected groups.

Hepatorenal and antioxidant indices results

The hepatorenal indices (AST, ALT, and Creat) were significantly increased (P<0.05) with higher MDA levels and lower CAT levels in the infected groups compared to the non-infected ones. Water exposure to 2.5-mg/L AILEE significantly (P<0.05) lowered the hepatorenal indices and MDA level and significantly increased the CAT level. Regarding the interaction between *A. veronii* infection and AILEE water exposure, the hepatorenal indices and MDA level were significantly increased (P<0.05) and lowered CAT levels in the infected fish treated with 0-mg/L AILEE compared to non-infected groups. By treating the infected fish with 2.5-mg/L AILEE, the hepatorenal indices and MDA level were significantly lowered (P<0.05) and modulated with increasing the CAT level (Table 4).

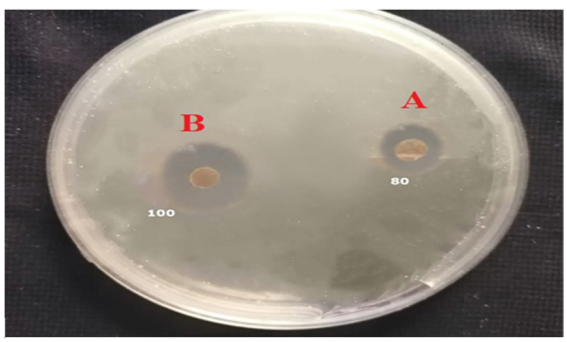

Fig. 1 Inhibition zone (mm) of AILEE against *A. veronii* at concentration of 80% (**A**) and 100% (**B**)

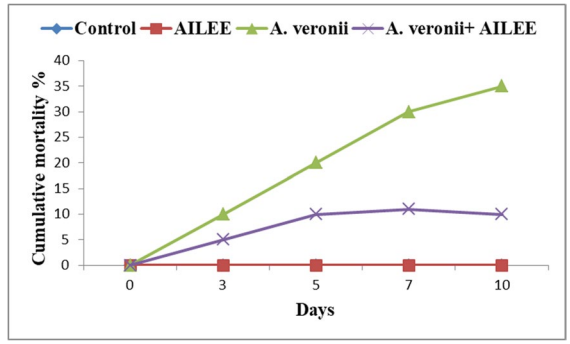

Fig. 2 Cumulative mortalities of *O. niloticus* challenged with *A. veronii* and treated with *Azadirachta indica* leave ethanolic extract (AILEE) as water exposure. Control and AILEE, fish groups non challenged and treated with 0- and 2.5-mg/L AILEE, respectively; *A. veronii* and *A. veronii*+AILEE, fish groups challenged with *A. veronii* and treated with 0- and 2.5-mg/L AILEE, respectively

Biochemical indices results

The protein profile indices (TP, ALB, and GLB) were significantly decreased (P<0.05) with increased stress indicators (GLU and CORT) (P<0.05) in infected groups compared to the non-infected ones. Water exposure to 2.5-mg/L AILEE had no significant effect (P>0.05) on the levels of protein profile indices while inducing a significant decrease (P<0.05) in the stress indicators compared to 0 mg/L AILEE exposure level. Regarding the interaction between *A. veronii* infection and AILEE water exposure, the infected group treated with 0-mg/L AILEE showed a significant decrease (P<0.05) in the levels of protein profile indices with higher stress indicators compared to other experimental groups. Treatment of the infected fish with 2.5-mg/L AILEE increased the protein profile and lowered the levels of GLU and CORT compared to the infected fish treated with 0-mg/L AILEE (Table 5).

Immunological indices results

The immunological indices (IgM, LYS, NO, and PA%) were significantly decreased (P<0.05) in the infected fish compared to the non-infected ones. Water exposure to 2.5-mg/L AILEE had a significant

Table 4 Effect of *Aeromonas veronii* and/or *Azadirchta indica* leave ethanolic extract (AILEE) on hepatorenal and antioxidant indices of *O. niloticus*

Effect of *A. veronii* infection	AILEE (mg/L)	AST (U/mL)	ALT (U/L)	Creat (mg/dL)	MDA (nmol/ml)	CAT (U/dL)
Effect of *A. veronii* infection						
Non-infected		10.21 ± 1.89^{b}	17.83 ± 1.44^{b}	0.31 ± 0.02^{b}	11.24 ± 3.90^{b}	29.88 ± 0.31^{a}
Infected		28.40 ± 1.52^{a}	39.10 ± 1.98^{a}	0.78 ± 0.01^{a}	50.56 ± 2.85^{a}	15.35 ± 0.25^{b}
Effect of AILEE						
0		22.69 ± 1.52^{a}	31.78 ± 1.44^{a}	0.60 ± 0.03^{a}	38.81 ± 3.93^{a}	20.30 ± 0.31^{b}
2.5		15.91 ± 1.33^{b}	25.15 ± 1.21^{b}	0.49 ± 0.02^{b}	22.99 ± 2.45^{b}	24.93 ± 0.32^{a}
Interaction						
Non-infected	0	10.54 ± 0.71^{c}	18.28 ± 1.02^{c}	0.30 ± 0.01^{c}	10.90 ± 0.40^{c}	27.49 ± 1.06^{b}
	2.5	9.88 ± 0.77^{c}	17.39 ± 0.85^{c}	0.32 ± 0.02^{c}	11.59 ± 0.71^{c}	32.28 ± 0.95^{a}
Infected	0	34.85 ± 1.50^{a}	45.28 ± 0.92^{a}	0.90 ± 0.01^{a}	66.73 ± 1.56^{a}	13.12 ± 0.42^{d}
	2.5	21.95 ± 1.74^{b}	32.91 ± 2.01^{b}	0.66 ± 0.05^{b}	34.39 ± 2.41^{b}	17.58 ± 0.70^{c}
Two-way ANOVA *P* value						
A. veronii infection		0.001	0.003	0.001	0.01	0.01
AILEE		0.001	0.01	0.001	0.02	0.01
Interaction		0.01	0.002	0.02	0.001	0.002

AST, Aspartate aminotransferase; *ALT*, alanine aminotransferase; *Creat*, creatinine; *MDA*, malondialdehyde; *CAT*, catalase. The values are mean ± *SE* ($N = 12$/group). Values in the same column that did not share the same superscripts are significantly different ($P < 0.05$; two-way ANOVA)

increase ($P<0.05$) in the immunological indices compared to 0-mg/L AILEE. Regarding the interaction between *A. veronii* infection and AILEE water exposure, the infected groups treated with 0 mg/L AILEE showed a significant decrease ($P<0.05$) in the levels of the immunological indices compared to the non-infected ones. While treating the infected group with 2.5-mg/L AILEE, the immunological indices significantly improved ($P<0.05$) (Table 6).

Discussion

Bacterial pathogen resistance to antibiotics is a huge problem facing the aquaculture industry globally and hinders the sustainable development of this sector. Finding safe and eco-friendly alternatives to antibiotics is a point of concern to reduce the problem of antibiotic resistance (Ghafarifarsani et al. 2023a). The plant extracts could replace antibiotics in treating bacterial diseases (Ghafarifarsani et al. 2023b; Yousefi et al. 2023a, b). *A. veronii* is a gram-negative bacteria producing serious clinical manifestations in fish like skin ulcers, petechial hemorrhage, and fin rot (Ibrahim et al. 2022a). AILEE exhibited in vitro antibacterial action against *A. veronii* with MIC and MBC of 40 and 50 µg/mL, respectively. The in-vitro antibacterial activity of AILEE was previously documented against Gram-negative bacteria like *Escherichia coli* and *Klebsiella pneumon*ia and Gram-positive ones as *S. aureus* (Adamu et al. 2019). The antibacterial activity of AILEE could be related to the detected active phytochemical compounds by the GC-MS analysis in this study. Our GC-MS results showed that AILEE had abundant polysaccharide compound [(D-(-)-fructofuranose, pentakis(trimethylsilyl) ether (isomer 2) (25.64%), D-(-)-fructofuranose, pentakis(trimethylsilyl) ether (isomer 1) (25.04%), D-fructose, 5TMS derivative (10.55%), D-(+)-talofuranose, pentakis(trimethylsilyl) ether (isomer 1) (6.23%), D-psicofuranose, pentakis(trimethylsilyl) ether (isomer 1) (4.26%), 3,8-dioxa-2,9-disiladecane, 2,2,9,9-tetramethyl-5,6-bis[(trimethylsilyl)oxy] (3.34%), arabinofuranose,

Table 5 Effect of *Aeromonas veronii* and/or *Azadirchta indica* leave ethanolic extract (AILEE) on Biochemical indices of *O. niloticus*

Effect of *A. veronii* infection	AILEE (mg/L)	TP (g/dL)	ALB (g/dL)	GLB (g/dL)	GLU (mg/dL)	CORT (ng/ml)
Effect of *A. veronii* infection						
Non-infected		4.14 ± 0.11^{a}	2.21 ± 0.15^{a}	1.92 ± 0.14^{a}	25.06 ± 1.89^{b}	4.72 ± 0.14^{b}
Infected		2.39 ± 0.12^{b}	1.17 ± 0.17^{b}	1.22 ± 0.15^{b}	48.23 ± 184^{a}	9.70 ± 0.13^{a}
Effect of AILEE						
0		3.08 ± 0.11	1.61 ± 0.17	1.47 ± 0.14	40.35 ± 1.89^{a}	8.87 ± 2.82^{a}
2.5		3.45 ± 0.12	1.77 ± 0.15	1.67 ± 0.11	32.95 ± 1.67^{b}	5.55 ± 1.95^{b}
Interaction						
Non-infected	0	4.13 ± 0.16^{a}	2.31 ± 0.55^{a}	1.81 ± 0.46^{a}	25.11 ± 2.83^{c}	2.53 ± 6.61^{c}
	2.5	4.15 ± 0.20^{a}	2.12 ± 0.13^{a}	2.03 ± 0.15^{a}	25.02 ± 2.30^{c}	2.19 ± 1.37^{c}
Infected	0	2.04 ± 0.08^{c}	0.91 ± 0.25^{c}	1.13 ± 0.25^{c}	55.58 ± 0.90^{a}	6.34 ± 1.18^{a}
	2.5	2.74 ± 0.30^{b}	1.42 ± 0.49^{b}	1.32 ± 0.48^{b}	40.88 ± 1.31^{b}	3.36 ± 2.27^{b}
Two-way ANOVA *P* value						
A. veronii infection		0.002	0.001	0.004	0.01	0.02
AILEE		0.13	0.53	0.33	0.01	0.001
Interaction		0.001	0.03	0.02	0.004	0.002

TP, Total proteins; *ALB*, albumin; *GLB*, globulin; *GLU*, glucose; *CORT*, cortisol. The values are mean ± *SE* ($N = 12$/group). Values in the same column that did not share the same superscripts are significantly different ($p < 0.05$; two-way ANOVA)

Table 6 Effect of *Aeromonas veronii* and/or *Azadirachta indica* leave ethanolic extract (AILEE) on immune indices of *O. niloticus*

Effect of *A. veronii* infection	AILEE (mg/L)	IgM (µg/mL)	LYS (ng/mL)	NO (mg/dL)	PA%
Effect of *A. veronii* infection					
Non-infected		11.86 ± 0.13^{a}	101.48 ± 1.89^{a}	27.24 ± 0.65^{a}	47.87 ± 0.74^{a}
Infected		4.26 ± 0.11^{b}	28.67 ± 1.58^{b}	18.38 ± 0.54^{b}	24.91 ± 0.54^{b}
Effect of AILEE					
0		7.12 ± 0.11^{b}	60.24 ± 1.19^{b}	20.63 ± 0.65^{b}	32.10 ± 0.74^{b}
2.5		9.00 ± 0.13^{a}	69.91 ± 1.20^{a}	25.00 ± 0.54^{a}	40.68 ± 0.57^{a}
Interaction					
Non-infected	0	11.01 ± 0.17^{b}	95.49 ± 2.04^{b}	24.10 ± 1.41^{b}	44.56 ± 1.22^{b}
	2.5	12.71 ± 0.50^{a}	107.49 ± 4.90^{a}	30.39 ± 1.31^{a}	51.19 ± 1.58^{a}
Infected	0	3.23 ± 0.10^{d}	25.00 ± 1.26^{d}	17.15 ± 0.61^{d}	19.65 ± 0.61^{d}
	2.5	5.30 ± 0.17^{c}	32.34 ± 0.87^{c}	19.60 ± 1.34^{c}	30.17 ± 2.19^{c}
Two-way ANOVA *P* value					
A. veronii infection		0.01	0.001	0.01	0.001
AILEE		0.02	0.002	0.01	0.002
Interaction		0.01	0.04	0.02	0.03

IgM, Immunoglobulin M; *LYS*, lysozyme; *NO*, nitric oxide; *PA%*, phagocytic activity%. The values are mean ± *SE* ($N = 12$/group). Values in the same column that did not share the same superscripts are significantly different ($p < 0.05$; two-way ANOVA)

1,2,3,5-tetrakis-O-(trimethylsilyl)- (2.18%), D-lyxose, 4TMS derivative (2.09%), D-xylopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)- (2.07%), and beta-D-galactofuranoside, ethyl 2,3,5,6-tetrakis-O-(trimethylsilyl)- (1.99%)]. Previous research had demonstrated that polysaccharides derived from herbal plants had antibacterial activities (Li et al. 2023; Wang et al. 2023; Yarley et al. 2021; Zhou et al. 2022). In addition, AILEE had alkaloid content (1,3-dihydroxyacetone dimer, 4TMS derivative)

(2.02%). The alkaloids exhibited an antibacterial activity through lysis and morphological changes of the bacterial cell wall (Sawer et al. 2005). Also, alkaloids are recognized as a DNA synthesis inhibitor by inhibiting topoisomerase (Guittat et al. 2003; Lisgarten et al. 2002). According to our GC-MS report, AILEE had malic acid, 3TBDMS derivative (3.24%) which possessed antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli O157:H7*, *Salmonella typhimurium*, and *Listeria monocytogenes* (Borah et al. 2023; Kim et al. 2016). In addition, AILEE had palmitic acid, TMS derivative (2.74%) and α-linolenic acid, TMS derivative (2.55%) which had previously been reported to exhibit antibacterial activity against *S. aureus* ATCC 6538, *E. coli* ATCC 8739, and *P. aeruginosa* ATCC 9027 (Morsi et al. 2023). The phytochemical analysis results in this study demonstrated that AILEE had various constituents (terpenoids, saponins, alkaloids, phenolics, tannins, glycosides, and flavonoids). Previous studies had proved the antibacterial actions of terpenoids (Banerjee et al. 2017; Chan et al. 2016; Sebei et al. 2015; Yang et al. 2020), saponins (Khan et al. 2018), phenols (Alibi et al. 2021; Takó et al. 2020), flavonoids (Xie et al. 2015), tannins (Karou et al. 2006), and glucoside (Parekh and Chanda 2007). The valuable biological constituents of AILEE were responsible for their antibacterial activity against *A. veronii.*

A. veronii infection produced severe clinical manifestations (skin hemorrhage, skin ulcer, and fin rot) and high mortalities in the challenged fish. The outcomes could be related to *A. veronii* virulence factors are blamed for the hemolytic and cytotoxic properties (Yang et al. 2021). Similar clinical signs were reported in Nile tilapia infected with *A. veronii* (Ibrahim et al. 2024; Reda et al. 2022). Rectifying the clinical signs and lowering the mortalities as a result of AILEE water exposure in the infected fish could be related to the antibacterial activity of the AILEE as a result of its biological constituents; resulting in prevention of the disease progression. Similarly, Hammed et al. (2017) found that AILEE had a therapeutic effect against *A. hydrophila* infection in African mud Catfish (*Clarias gariepinus*). A similar ameliorative effect was reported in Nile tilapia infected with *A. sobria* and treated with chitosan neem nanocomposite (Ibrahim et al. 2023). Additionally, *A. indica* aqueous extract relieved the clinical symptoms in Nile tilapia infected with *Citrobacter freundii* (Thanigaivel et al. 2015).

Hepatic enzymes (ALT and AST) are indexes for liver function. As well as Creat is an indicator of kidney function (Abdel Rahman et al. 2023a, b). MDA is an indicator of tissue damage; higher MDA level and lower CAT activity indicate an oxidative stress condition (Ahmed et al. 2021). Elevated hepatorenal indexes with higher MDA level and lower CAT activity ($P<0.05$) as a result of *A. veronii* infection indicate hepatorenal dysfunction and oxidative stress. Elevated hepato-renal function indicators and oxidative stress were reported in *C. gariepinus* as a result of *A. veronii* infection (Mahboub et al. 2024). The elevated hepatorenal indices caused by *A. veronii* infection could be attributed to damaged hepatocytes and renal cells by *A. veronii* virulence components, increasing these indicators in the blood (El Latif et al. 2019). Amelioration of the hepato-renal functions and MDA level with improved CAT activity ($P<0.05$) was evident in the *A. veronii* infected fish when treated with AILEE. These outcomes could be related to the improvement of the antioxidant activity which was obvious in this study by AILEE therapy. The antioxidant activity of AILEE was approved in this study by the DPPH activity (77%), as well as, the antioxidant and hepatoprotective effects of some AILEE biological constituents like phenolics, flavonoids, terpenoids, saponins, alkaloids, tannins, and glycosides (Akanitapichat et al. 2010; Alqasoumi and Abdel-Kader 2012; Jia et al. 2019; Kinoshita et al. 2007; Oh et al. 2004; Vijayan et al. 2003; Xiao et al. 2016).

Blood elements including GLU and CORT gauge a fish's well-being and stress levels (Abarra et al. 2017). The primary source of energy for fish to withstand unfavorable conditions is blood GLU, a trustworthy indicator of stress (Fazio et al. 2015). Elevated GLU and CORT levels ($P<0.05$) due to *A. veronii* infection could be related to the stress condition caused by infection. Similarly, Hashem et al. (2022) documented that *A. veronii* induced an elevation in the serum oxidative stress markers (GLU and CORT) in Nile tilapia. Modulation ($P<0.05$) of the GLU and CORT levels in the *A. veronii* infected fish and treated with AILEE indicated the anti-stress properties of AILEE due to its active biological constituents. The hypoglycemic effect of AILEE was previously reported (Mukherjee and Sengupta 2013; Pingali et al. 2020).

Fish immune parameters are crucial barometers for assessing fish health; non-specific immunity is the cornerstone of fish immunity against all infections and assists in the development of the adaptive immune response (Ibrahim et al. 2022b). Blood proteins, in particular GLB, are assumed to reflect the fish's improved immunological response (Sahoo et al. 2021). IgM is the main kind of systemic immunoglobulin that is often seen in fish serum. IgM defends the fish from infection by several of mechanisms, including neutralization, complement system activation, and phagocytosis (Jones et al. 2022). LYS, NO, PA, and TP are used for assessing innate immune functions (Abdel Rahman et al. 2022).

A. veronii infection resulted in immune-suppression through lowering the immune indices (TP, ALB, GLB, IgM, LYS, NO, and PA) ($P<0.05$) in the infected fish. Similar immune-suppression was reported in Nile tilapia (Ibrahim et al. 2024) and *C. gariepinus* (Mahboub et al. 2024) as a result of *A. veronii* infection. Improving the immune indices ($P<0.05$) of the infected fish after water exposure to AILEE could be related to the immunostimulant properties of the biological constituents of AILEE. The AILEE active constituents that had immunostimulant properties like phenolics, flavonoids, terpenoids, saponins, alkaloids, tannins, and glycosides (Leite et al. 2022; Li et al. 2019; Liu et al. 2012; Peng et al. 2021; Škubník et al. 2021; Ye et al. 2019). In addition, our GC-MS results revealed that AILEE had malic acid, 3TBDMS derivative (3.24%) which improved the antioxidant and immune responses of *O. mykiss* (Yousefi et al. 2023a, b). Additionally, palmitic acid, TMS derivative (2.74%) which was detected in our GC-MS analysis was previously reported to have an immunostimulatory effect in zebrafish (*Danio rerio*) (Librán-Pérez et al. 2019). Similarly, α-linolenic acid, TMS derivative (2.55%) was reported to have an immunostimulatory effect in Nile tilapia (Chen et al. 2016) and grass carp (*Ctenopharyngodon idella*) (Kong et al. 2019).

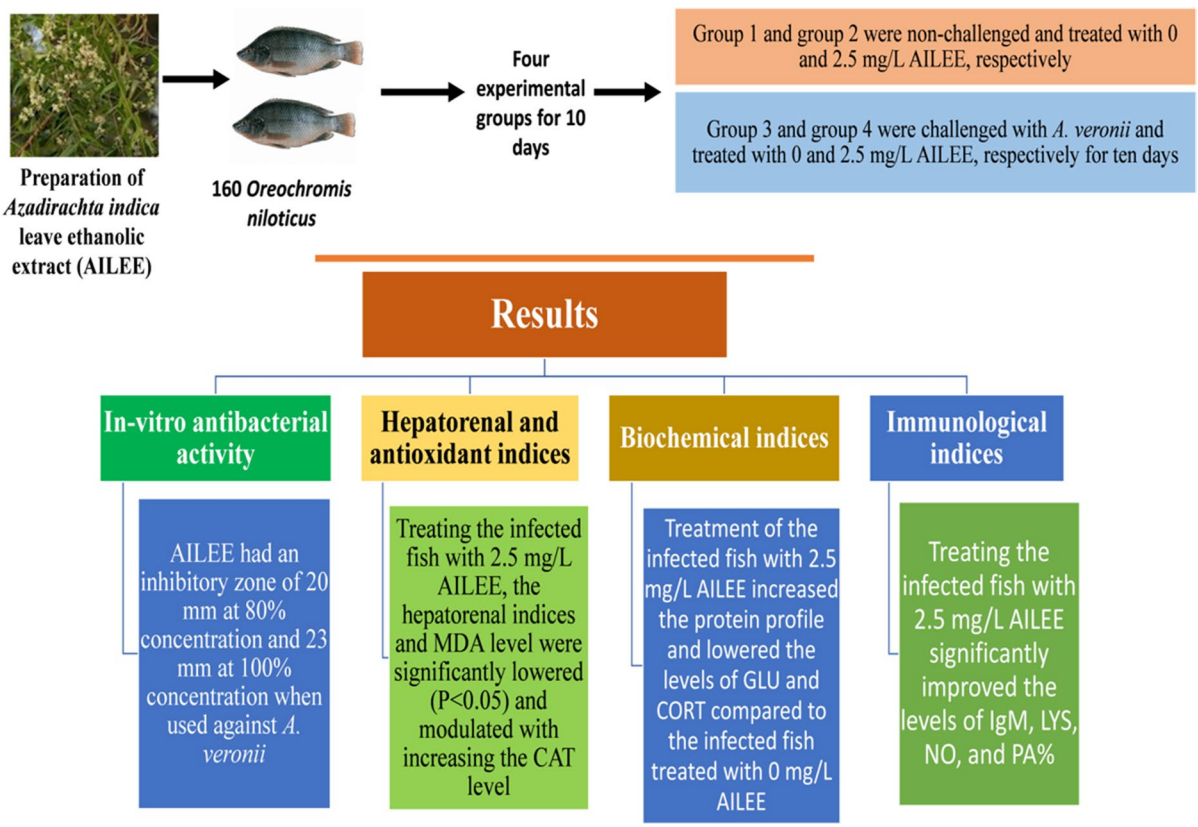

Fig. 3 Summary for the study results

The main limitations of the current study design are the considerably short duration of the trial, and the therapeutic effect of AILEE as feed additive was not studied. Study with a longer duration and including AILEE as a therapeutic feed additive is designated.

Conclusion

Controlling bacterial outbreaks and prohibiting the use of antibiotics in aquaculture are difficult tasks. That's why safe alternative protocols are sorely needed. The results of the study (Fig. 3) indicate that AILEE had therapeutic activities against *A. veronii* infection in *O. niloticus*. AILEE as water exposure modulates the hepatorenal dysfunction and stress biomarkers, as well as improving the immune/antioxidant indices in *A. veronii* infected fish. AILEE is a promising therapeutic agent that can be used in mitigating bacterial pathogens in aquaculture systems. The results of this investigation help the sustainable development of Nile tilapia through controlling *A. veronii* worth consequences and reducing the mortalities as a result of this infection. In addition, AILEE provides an eco-friendly alternative to antibiotic use in fish farms, consequently aiding in reducing the antibiotic resistance problem.

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Data availability statement All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval The Institutional Animal Care and Use Committee of Zagazig University, Egypt, approved the experimental protocol (ZU-IACUC/2/F/136/2021), and all applicable institutional standards were followed when caring for and using animals in this study.

Consent to participate All authors have participated in this work.

Consent for publication All authors review and approve the manuscript for publication.

Competing interests The authors declare no competing interests.

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