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Original Article

The Role of Laminarin in improving Inflammatory, oxidative stress marker, and liver function against *Aeromonas hydrophila* toxicity

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Abstract

Background: *Aeromonas hydrophila,* originally named Bacillus hydrophilus fuscus, was the first discovered species of Aeromonas. It is a gram-negative bacterium that is widespread in inland aquatic environments, benthic sediments, and other aquatic organisms. Although it is an emergent bacterial pathogen in humans and aquaculture, it is part of the normal intestinal microflora of healthy fish and is considered an opportunistic agent. **The aim of the study:** Effect of the fungal cell wall, Laminarin (β-1,3-D-glucans), on some biochemical constituents in the blood and liver of the albino rats (*Rattus norvegicus*) was determined. **Material and Method:** Rats were intraperitoneally injected with 0.2 ml of $1x10^7$ *Aeromonas hydrophila* suspended in 0.9% saline solution every day for eighteen days. Another group of rats was injected (i.p.) with 0.2 ml of the same bacterial concentration for six days and then injected with 0.2 ml of 15 mg/100 b.wt of Laminarin at different time intervals. **Result:** Daily observations of the animals after their injection with *A. hydrophila* showed gradual paleness of the eyes during the period of injection; normal dark red-colored eyes turned gradually to clearly pale red after 15 days. The estimation of liver function, inflammatory and oxidative stress markers in serum as well as liver homogenate showed increases in their levels during almost all time intervals after bacterial infection, while laminarin enhanced almost all these values. **Conclusion:** The administration of laminarin helps improve of *Aeromonas hydrophila* toxicity. In addition, the symptomatic changes recovered to approximately normal status at all different times.

Keywords: Laminarin, liver function, oxidative stress markers.

Introduction

Aeromonas and Pseudomonas are facultative bacteria, found everywhere, and have pathogenic potential for fish. These bacteria are found in fish hatcheries and farms, and can easily develop colonies on the skin, fins, gills, and gut of fish. The most common diseases in freshwater are abdominal dropsy, columnaris, furunculosis, tail-rot, and fin-rot (1). Recent studies claim A. hydrophila to be the most common freshwater bacteria integrated with disease exposure and cause of economic loss for the aquaculture industry worldwide (2).

A. hydrophila is responsible for causing the abdominal dropsy affecting many species of fish in the Indian subcontinent (3). A. hydrophila causes an acute type of disease in fish, showing clinical symptoms such as severe abdominal dropsy, scale protrusion, sore, spotting on the body, blisters, and chronic ulcerous type with furuncles (4). In intensive culture systems, fish suffer from abdominal dropsy due to A. hydrophila occurring in

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the monsoon season, and 80% is due to rough handling and stress (5).

The pathogenicity of *A. hydrophila* has been investigated in diverse fish species, which mainly resulted from heterogenic strain and differences in enterotoxin and adhesive mechanisms accountable for the outbreak of infection in fish aquaculture (6). Antibiotics are thought to be harmful for aquatic life poising environmental risks and resulting in antibiotic resistance. The synthetic chemicals and antibiotics are used in Pakistan for the prevention of fish diseases, which may result in the appearance of antibiotic-resistant microbes, drug residues, and environmental hazard effects (7).

Aeromonas hydrophila is the major pathogen of cultured sturgeon species, and in some cases, the mortality even reaches 100% (8). *A. hydrophila* is a facultative anaerobic, gram-negative, rod-shaped bacterium occurring in all bodies of water worldwide (9). Outbreaks of *A. hydrophila* infectious diseases have been identified in various countries and previously well described as the pathology causing septicemia with an open dermal ulcer, gastrointestinal hemorrhage, ascites, and cloacal hemorrhaging (10 - 13).

However, the symptoms and pathological lesions in infected fish differ depending on the particular bacterial isolates or strains. The ability of this bacterium to cause disease depends largely on the presence of various virulence factors, which include aerolysin (aer), serine protease (ser), elastase (ahyB), cholesterol acyltransferase (gcaT), type III secretion system (ascV), DNases (exu), polar flagella (fla), cytotonic enterotoxins (act, alt, ast), and lipase (lip) (14 – 16). It was reported that aerolysin, cytotoxic enterotoxin, and extracellular serine protease are the most critical genes for identifying potentially pathogenic *A. hydrophila* strains (17, 18).

However, the data available in this respect indicate that the pathogenicity of *A. hydrophila* is probably multifactorial and does not arise from a single gene, but it is likely the result of synergistic effects of several virulence genes (19 -22). Most of the sturgeon species in Kazakhstan are cultured, and bacterial pathogens are the main cause of mortality in these fish (23, 24); however, to date, information about diseases and fish health control is rather limited. To our knowledge, there is no record of *A. hydrophila* infection among cultured sturgeons in Kazakhstan.

Laminarin: Saccharina and Laminaria species, which are popular origins of dietary fiber in various nations, are utilized to make the storage glucan known as laminarin. Laminarin also has potent biofunctional properties, including anti-tumor and anti- apoptotic properties (25).

Material and Methods

1. Animals

Forty eight Adult male Wistar rats (Animal Care Unit, Faculty of Agriculture, Minia University, weighing (180 – 200 g) Before the trial began, the animals were housed in conventional laboratory conditions (24-hour cycles of light and darkness, 42-53 percent relative humidity, and a temperature of 22-24 °C), given tab water, and allowed to acclimate for a 10 days.

2. Media

Bacteria were grown on nutrient agar (pH 6.8) and nutrient broth (pH 7.5–7.6) (26, 27). The bacterial identification was performed, and the concentrations of bacteria were calculated using the matching technique developed by *McFarland* after some modifications (28, 29).

3. Laminarin

Laminarin approx. 95% from cell walls of *Laminaria digitata*, was purchased from Sigma (England), suspended in saline solution (1% W/V) and administered at 0.2 ml of 1% laminarin (15 mg/100 gm b.wt.) at different time intervals.

4. Experimental Design

Group I (Control): This group was injected with an equivalent dose of saline solution.

Group II (Infected): the infected group was injected intraperitoneally with 0.2 ml of bacterial

suspensions containing $1x10^7$ cells/ml of NaCl (0.9%).

Group III (Treated): the treated group was intraperitoneally injected daily with 0.2 ml of bacterial suspensions containing $1x10^7$ cells/ml of NaCl (0.9%) for six days and then injected with 0.2 ml of 1% laminarin (15 mg/100 gm b.wt) at different time intervals. Five rats were sacrificed after 6^{th}, 9^{th}, 12^{th}, 15^{th}, and 18^{th} days, respectively.

5. Blood sample collection

Animals were sacrificed by decapitation, and the blood was first collected in clean centrifuge tubes, allowed to clot for 30 minutes at room temperature, and then centrifuged at 4000 rpm for 10 minutes (Z 200 A, Hermle, Germany) to obtain the sera (supernatants), which were preserved at -80 C immediately after separation for biochemical analysis.

6. Biochemical examination

Alanine aminotransferase (ALT), aspartate aminotransferase (AST). Alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT), albumin, and total proteins colorimetric assay kits were purchased from Spectrum-diagnostics, Cairo, Egypt.

7. Oxidative stress markers determination

Malondialdehyde (MDA) and reduced glutathione (GSH) colorimetric assay kits were obtained from Biodiagnostic, Giza, Egypt.

7. Inflammatory markers determination

Tumor necrosis factor-alpha (TNF-α) and Interleukin (IL-6) ELISA kits were purchased from Elabscience, Texas, USA.

8. Homogenization of rat liver

0.5 gm of rat liver was homogenized in 5 mL of ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA using a homogenizer (30). The homogenate was centrifuged at 3000 rpm for 10 minutes. The obtained supernatant was then diluted 10 times with the same buffer.

9. Statistics

Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) program version 26.0. Results were shown as mean ± standard deviation (SD). Differences with $P<0.05$ were accepted as significant, differences with $P<0.01$ or $P<0.001$ were considered as moderately or highly significant, respectively.

RESULTS

1-Symptomatic changes

Daily observations of the animals after their injection with *A. hydrophila* showed gradual paleness of the eyes during the period of injection; normal dark red-colored eyes turned gradually to clearly pale red after 15 days. Also, the animal's skin showed considerable shrinking. It was also noted that the animal's temperature raised (fever) and the feces consistency and color changed gradually from solid brownish black feces into nearly liquid yellowish brown, indicating the occurrence of diarrhea. Finally, this diarrhea was associated, especially among the last three time intervals, with loss of appetite, which had led to loss of body weight and activity.

2-Biochemical studies

In table (1), The ALP, GGT. ALT and AST activities in serum and ALT and AST in liver (Table 3) increased significantly in bacterial infected groups. The highest value of liver function enzyme was recorded after 18 days of infection compared with the control group. Laminarin treated group showed a significant decrease in the liver function enzyme. (Tables 1, 3).

The concentration of albumin and total protein in serum and liver of bacterial-infected groups showed a significant ($P < 0.05$) increase after all time periods of infection. The maximum increase was achieved after 18 days (serum 7.8±0.6 gm/dl, 8.7±0.7 gm/dl, 0.178±0.5 gm/gm, 0.19±0.5, respectively) compared to the control value (Tables

2, 3). On the other hand, laminarin changes the total protein values to approximate the level of the control value.

In Table 4, the *Aeromonas hydrophila* group showed a highly significant (P>0.001) increase in MDA and decreased levels of GSH compared to the control groups. Post-treatment with laminarin reversed the levels of oxidative stress markers.

The inflammatory markers were impacted before and after *Aeromonas hydrophila* injection in rats, as shown in Table 5. The *Aeromonas hydrophila* exhibited highly significant (P>0.001) elevated serum levels of TNF-α and IL-6 compared to the healthy groups. But administration of laminarin indicated a highly significant (P>0.001) decrease in the levels of TNF-α and IL-6 in the treatment groups compared to the *Aeromonas hydrophila* group.

Table 1 represents the mean value of liver function in serum

		Time (Days)				
Parameters	Group	6	9	12	15	18
ALP (U/L)	Control	75.6±0.77	74.3±0.68	76.4±0.55	75.4±0.47	73.2±0.58
	A. Hydrophila	87.3*±0.5	91.4*±0.4	103.9*±0.6	112.2*±0.6	118.6*±0.7
	Laminarin	82.5±0.4	89.2*±0.5	99.1±0.4	89.5±0.6	88.8±0.7
GGT (U/l)	Control	9.2±0.87	9.2±0.87	9.2±0.87	9.2±0.87	9.2±0.87
	A. Hydrophila	12.4*±0.2	14.9*±0.4	15.6*±0.5	18.9*±0.5	21.9*±0.6
	Laminarin	11.8±0.4	13.5±0.5	13.2±0.6	15.7±0.8	15.4±0.6
ALT (U/L)	Control	27.3±0.6	27.5±0.3	28.8±0.5	38.7±0.6	39.8±0.6
	A. Hydrophila	42.3*±0.3	47.2*±0.6	51.3*±0.7	56.5*±0.8	55.2*±0.5
	Laminarin	29.3±0.3	28.8±0.4	31.2±0.2	35.7±0.6	29.8±0.2
AST (U/L)	Control	24.2±0.4	26.4±0.3	27.5±0.4	25.7±0.4	27.4±0.2
	A. Hydrophila	36.8*±0.1	40.4*±0.9	42.4*±0.3	47.5*±0.2	51.1*±0.6
	Laminarin	26.3±0.4	28.5±0.4	31.2±0.2	34.4±0.5	31.2±0.5

Table 2 represents the mean value of Albumin and Total Protein in serum

		Time (Days)				
Parameters	Group	6	9	12	15	18
Total protein (gm/dl)	Control	5.3±0.3	5.2±0.6	5.7±0.6	5.9±0.3	5.8±0.8
	A. Hydrophila	7.8*±0.3	8.4*±0.7	7.9*±0.6	8.6*±0.4	8.7*±0.7
	Laminarin	5.9±0.4	4.6*±0.4	7.1±0.7	5.7±0.6	7.8±0.7
Albumin (gm/dl)	Control	4.5±0.1	4.3±0.5	4.8±0.3	4.4±0.3	5.1.±0.4
	A. Hydrophila	6.4*±0.2	6.7*±0.5	6.5*±0.6	7.7*±0.6	7.8*±0.6
	Laminarin	4.9±0.4	4.3±0.4	5.3±0.4	4.6±0.5	5.5±0.2

Table 3 represents the mean value of Albumin, Total Protein, AST, and ALT in the liver

		Time (Days)				
Parameters	Group	6	9	12	15	18
Total protein (gm/gm)	Control	0.14±0.5	0.15±0.1	0.14±0.8	0.15±0.5	0.14±0.5
	A. Hydrophila	0.17*±0.5	0.18*±0.6	0.18*±0.7	0.19*±0.4	0.19*±0.5
	Laminarin	0.14±0.3	0.14±0.4	0.15±0.4	0.15±0.5	0.14±0.5
Albumin (gm/gm)	Control	0.108±0.3	0.119±0.6	0.137±0.6	0.147±0.7	0.150±0.8
	A. Hydrophila	0.141*±0.5	0.142*±0.6	0.173*±0.3	0.177*±0.3	0.178*±0.5
	Laminarin	0.115±0.5	0.127±0.4	0.143±0.4	0.151±0.7	0.147±0.4
ALT (U/gm)	Control	0.77±0.5	0.79±0.4	0.76±0.4	0.75±0.6	0.77±0.5
	A. Hydrophila	0.84±0.1	0.86*±0.1	1.1*±0.3	0.89*±0.1	0.85*±0.2
	Laminarin	0.74±0.5	0.67*±0.4	0.74±0.4	0.75±0.6	0.77±0.24
AST (U/gm)	Control	0.62±0.4	0.71±0.6	0.75±0.2	0.80±0.6	0.68±0.4
	A. Hydrophila	0.82*±0.5	0.84*±0.6	0.91*±0.5	0.99*±0.8	1.3*±0.7
	Laminarin	0.61±0.4	0.67±0.3	0.71±0.5	0.77±0.5	0.76±0.3

Table 4 represents the mean value of serum oxidative stress

		Time (Days)				
	Group	6	9	12	15	18
GSH (mmol/ml)	Control	3.3 ± 0.7	3.2 ±0.7	3.3 ± 0.4	3.8 ± 0.5	3.8 ± 0.2
	A. Hydrophila	2.5*±0.4	2.6*±0.8	1.3*±0.6	1.7*±0.2	1.9*±0.5
	Laminarin	2.6±0.3	2.8±0.2	2.9±0.6	3.1±0.5	3.6±0.5
MDA (mmol/ml)	Control	0.75 ± 0.4	0.74 ± 0.5	0.72 ± 0.7	0.70 ± 0.5	0.75 ± 0.4
	A. Hydrophila	2.1*±0.7	2..8*±0.7	3.1*±0.2	3.5*±0.8	3.9*±0.6
	Laminarin	1.9±0.4	2.1±0.3	1.9±0.5	1.5±0.4	0.99±0.2

Table 5 represents the mean value of serum inflammatory markers

		Time (Days)				
	Group	6	9	12	15	18
TNF-α (pg/ml)	Control	68.3± 1.7	69.3± 1.1	67.3± 0.7	66.4± 1.5	69.7± 1.2
	A. Hydrophila	74.5*±0.4	75.6*±0.8	78.3*±0.6	88.7*±0.2	96.4*±0.5
	Laminarin	72.1±0.3	71.2±0.2	70.8±0.6	69.7±0.5	68.4±0.5
IL-6 (pg/ml)	Control	10.6 ± 0.5	10.7 ± 0.4	11.3 ± 0.6	13.3 ± 0.6	12.3 ± 0.8
	A. Hydrophila	12.1*±0.7	14..9*±0.5	15.6*±0.8	16.7*±0.9	18.9*±0.9
	Laminarin	12.2±0.4	13.1±0.3	13.9±0.5	15.5±0.4	16.2±0.2

Discussion

Aeromonas hydrophila has become one of the most substantial threats to the tilapia industry; a previous study reported that its prevalence ranges from 3.33% to 46.66% on Brazilian fish farms (31). Although antibiotics have often been used to control infections, the use of antibiotics leads to the accumulation of drug residues in animals and the evolution of resistance in pathogenic bacteria (32). Early surgical debridement and antimicrobial therapy are recommended, but rapid worsening often results in death (33); so, we have to pay attention to infection of *Aeromonas hydrophila,* especially in patients with liver cirrhosis.

The liver plays a vital role in protein metabolism. Results from animal studies have shown profound changes in the rate of protein synthesis of the liver in response to trauma and critical illness, including enhanced synthesis of acute-phase proteins (34,35). This may represent a reason for the protein changes in this study. In this study, it was reported that the elevation of liver enzyme activities causes liver and kidney damage, and a higher ALP level in blood leads to alterations of enzymes, causing skeletal disorders that include osteoporosis and hepatic cell ruptures (36).

The effect of stress bacterial infection caused the secretion of stress hormones, cortisol, and thyroid hormones, which may be a reason for the metabolic changes that occurred in the liver and serum contents of protein, albumin, oxidative marker, inflammatory marker, and liver enzyme. On the other hand, the effects of laminarin administration may be mediated through receptor binding and subsequent cell activation. In addition, the modulatory effect of laminarin on metabolic pathways in the liver and serum may be explained as a result of its effect on hormonal secretion as an integral part of the metabolic mechanisms. Laminarin: Saccharina and Laminaria species, which are popular origins of dietary fiber in various nations, are utilized to make the storage glucan known as laminarin. Laminarin also has potent biofunctional properties, including anti-tumor, antioxidant, and anti- apoptotic properties (25).

Lipopolysaccharides (LPS) are surface bacterial toxins capable of eliciting a wide variety of pathological effects, such as shock, tissue injury, and death in humans and animals. Therefore, LPS may represent another reason for the previously described symptomatic changes. However, production of cytotoxin and haemolysin was prominent amongst isolates from various clinical sources and may be an inherent characteristic of *A. hydrophila* irrespective of the source of specimen. Haemolysin and cytotoxin activity were reported to be common in extra-intestinal isolates, which might signify the importance of these toxins in the pathogenesis of *Aeromonas* infection (37).

laminarin, a β-(1,3) glucan, is represented as a specific node from glucan, and many studies have emerged exhibiting its potential as an anti-inflammation agent, in cancer therapy, and in tissue engineering (25). Interestingly, cellulose is decoupled from glucans with a separate and emerging node without any interconnections. Specific nodes for biomedical applications of macroalgal polysaccharides are also noted. Separate nodes for anti-neoplastic, anti-allergic, anti-inflammatory, anti-bacterial, and antihypertensive agents show the emerging importance of algal polysaccharides.

A. hydrophila increased haemolytic and proteolytic damage of host tissue by *A. hydrophila,* which causes the liberation of intracellular iron stores for use by the organism during in *vivo* growth (38). One could speculate that the increased protein, albumin, and enzymes (ALT and AST) in both serum and liver might have been due to the previous destructive effects of bacteria and their toxins.

Also, the proteases reduce the clotting time of blood and induce an alteration in the membrane lysophospholipid content, which can affect the activity of membrane-bound enzymes, causing RBCs to lyse. This may account for the increased protein and enzymes in both liver and serum described in the present study after *A. hydrophila*

infection. However, the increase in protein contents and enzyme activities at the time intervals (39, 40). Mortality recorded in this study may be due to: (1) the proteases secreted by *A. hydrophila* break down the serum lipoproteins to insoluble products (2) the interaction between the serum components and the extracellular hemolytic toxin which results in net activation of phospholipase and hemolytic activities of the toxin and causes disturbances in the properties of the serum albumin and lipoproteins and hence increases either total protein content or enzymes and albumin content in liver and serum (41, 42).

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There is no source of funding for this study.

Conflicts of Interest

The author declares that there are no conflicts of interest.

Ethical approval

The experiment and rats handling were carried out as stated by the guidelines of the Committee of Research Ethics of Faculty of Pharmacy, Minia University, Egypt **(MPEC-2305113)**, and followed the principles outlined in the Guide for the Care and Use of Lab Animals. Every attempt was made to reduce animal suffering and use as few animals as possible.

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