Zagazig Veterinary Journal, ©Faculty of Veterinary Medicine,
Zagazig University, 44511, Egypt.
Volume 50, Number 4, p335-349, December 2022
DOI: 10.21608/zvjz.2022.179715.1198

RESEARCH ARTICLE

The Immuno-Antioxidant and Anti-bacterial Effects of Clove Powder on *Proteus mirabilis* Challenge in *Oreochromis niloticus*: A Comparative Study with Cephalexin Antibiotic

Zeinab El-Bouhy[1], Afaf N. Abdel Rahman[1], Mohammed Wahbah[2] and Shaimaa A.A. Ahmed[1*]
[1] Department of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, P.O. Box 44511, Zagazig, Sharkia, Egypt
[2] Veterinary Administration, Aga, Dakahlia Governorate, Egypt
*Corresponding Author: shaimaakarim2020@gmail.com

Article History: Received: 13/12/2022 Received in revised form: 25/12/2022 Accepted: 31/12/2022

Abstract

Currently, herbal therapy has become an important alternative that is widely used in aquaculture to limit the use of antibiotics and other chemicals during fish production cycle. Correspondingly, the ongoing study was performed to evaluate the therapeutic efficacy of clove powder (CP) (*Syzygium aromaticum*, L.) as an aqueous additive in comparison to cephalexin (CE) antibiotic in Nile tilapia (*Oreochromis niloticus*, L.) experimentally challenged with *Proteus mirabilis* (PM). Nile tilapia (*N* = 240) were allocated into eight groups, each group had three replicates. The first group (control) was neither challenged, nor treated. Fish of the second and third groups (CP1 and CP2) were maintained in water containing CP at 0.01 and 0.02 g/L, respectively .Meanwhile, the fourth group (CE) was exposed to CE at a concentration of 0.5 g/L. The fifth group (PM) served as a positive control (challenged with *P. mirabilis* (5×10^6 CFU/mL) and non-treated). Meanwhile, the other three groups (CP1-PM, CP2-PM, and CE-PM) were intraperitoneally challenged with *P. mirabilis*, then treated with medical bath containing either CP or CE with the same concentrations as previously mentioned. The experiment lasted for 15 days, during which all fish were kept under observation. Following the challenge by *P. mirabilis*, the highest mortality rate (85%), notable clinical symptoms, elevated stress (glucose and cortisol) and hepato-renal function (alanine and amino transferases, urea, and creatinine) indicators were observed. Marked decline ($P< 0.05$) in the immunological response (lysozyme and nitric oxide) and antioxidant biomarkers (catalase, superoxide dismutase, and reduced glutathione content) were noted in the PM group. Surprisingly, a considerable improvement in all these indices with reduction in the mortality was noticed in *P. mirabilis*-challenged groups that treated with either CP2or CE (30% and 20% respectively).Thus, we recommend the usage of CP (0.02 g/L) as a natural, potent immuno-stimulant, and antibacterial agent alternative to antibiotics to avoid their negative impacts and pave the way towards a sustainable aquaculture industry.

Keywords

Nile tilapia; Therapeutics; *Syzygium aromaticum*; *P. mirabilis*; Immune response

Introduction

Being the main source of fish protein; nowadays the size of international investments of fisheries and aquaculture outputs has witnessed an outstanding growth and wide expansion all over the world [1]. Owing to its high digestibility and rich content of several essential amino acids, the aquatic protein of seafood and other aquatic animal products is considered a very rich source for high quality protein surpassing the terrestrial protein producing animals [2]. Nile tilapia (*Oreochromis niloticus*, L.)is among the most important cultured fishes worldwide; this stems from its outstanding aquaculture traits including high growth rates and tolerance to adverse environmental conditions [3,4].

Although the intensive system for fish farming is accompanied by multiple drawbacks, it was approved to be the suitable practice to fulfill the increasing demands for fish. Bacterial diseases representing one of the major drawbacks occur in cultured *O. niloticus*, mainly when farmed under high stocking densities; thus leading to high mortalities of fish and consequently enormous economic losses in fish farms [5]. *Proteus* species is Gram negative, facultative anaerobic rods, which belong to family *Enterobacteriaceae*. *Proteus* species especially, *Proteus mirabilis* has been recognized as a potential fish pathogen. In fish, it is a reliable sign of sewage pollution because it inhabits both human and animal intestines [6]. Earlier, different *Proteus* spp. caused mortalities in Nile tilapia, red swamp crayfish (*Procambarus clarkia*, L.) [7] and koi carp (*Cyprinus carpio koi*, L.) [8]. Additionally, it was noted that the Indian main carp (*Labeo rohita*, L.) was infected by *P. mirabilis* and developed disease symptoms such as hemorrhages on all body surfaces, histological lesions in key crucial organs, and mortalities [9]. The pathogenicity and impacts of *P. mirabilis* are attributed to the bacterium's ability to swarm on solid surfaces, as well as the action of various virulence factors produced by the bacteria, such as extra-cellular protein, somatic antigens, colicins, and lipopolysaccharides [10].

Along the last few decades, treatment of fish bacterial disease depended mainly on the usage of antibiotics; that exert their action either through killing micro-organisms or inhibiting their growth. However, the excessive usage of these antibiotics in treating various disease problems of fish led to several negative impacts including emergence of new bacterial generations with ability to resist antibiotic therapy, suppression of the immune response, destruction of the beneficial bacterial population in the aquatic environment in addition to the accumulation of those antibiotic residues in the environment and/or fish tissues [11, 12]. Hence, all these disadvantages were the major impetus for developing effective, eco-friendly alternative strategies for antibiotics [13, 14].

Owing to their powerful traits as immune-stimulators, antioxidants, growth promoters, natural plants and/or their extracts were used as antibiotic alternatives in aquaculture practices [15]. Clove (*Syzygium aromaticum*, L.) is among the most influential antimicrobial medicinal herbs; this is because of its rich content of multiple bioactive phenolic compounds including flavonoids, hydroxycinnamic acids and hydroxybenzoic acids hydroxyphenyl propene. Although eugenol is the major bioactive component of clove, some other constituents present in lower concentrations like quercetin and kaempferol [16]. These bioactive constituents enable the clove to act as an antioxidant, antiviral, antimicrobial, and anticancer agent [17]. The information on utilizing clove as a therapeutic agent for fish, however, is still lacking. Therefore, the ongoing investigation was performed to elucidate the impact of clove powder (CP) on immunity, hepato-renal function biomarkers, antioxidant status together with evaluating its antibacterial activity against *P. mirabilis* infection in Nile tilapia.

Materials and methods

Medicinal plant preparation

Clove buds were procured from Giza seeds and herbs Company, Cairo, Egypt. The buds were air-dried, then ground to a fine powder to be used.

Bacterial strain

Proteus mirabilis isolate used in this challenge was previously isolated from naturally infected Nile tilapia, at the laboratory of Department of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Egypt (unpublished data). This isolate was cultured onto tryptic soy agar (TSA)

(Himedia, India) at 28 °C for 24 h then was identified by biochemical tests, VITEK® 2 system (BioMérieux Inc., NC, USA) and *hpmA* gene using the following oligonucleotide primer pair sequences; F: CCAGTGAATTAACGGCAGGT and R: CGTGCCCAGTAATGGCTAAT giving an amplicon of 654 bp [9]. It was approved to be pathogenic through re-isolation from fish experimentally infected with the same isolate.

The lethal dose (LD_{50}) of *P. mirabilis* was determined according to Abdel-Razek*et al.* [18] and it was 10^7 CFU/mL; while a sub lethal dose, 0.1 mL of 24-h broth (5×10^6 CFU/mL) was used for the challenge test [19].

In vitro sensitivity testing of the antimicrobial agents and clove powder

Antimicrobial susceptibility of *P. mirabilis* was tested against 16 commercially available antimicrobial discs (Oxoid, Engeland) using the disc diffusion method according to Blair*et al.* [20] as shown in Table 1. Additionally, for investigation of *P. mirabilis* sensitivity to CP; different concentrations of CP were dissolved in distilled water (0, 2.5, 5, 10, 20, and 40 µg mL^{-1}), then sterile discs were soaked in the dissolved powder following the methods of Tshabalala *et al.* [21]. Subsequently, one hundred µL of *P. mirabilis* cell suspension was spread on a nutrient agar plate; the discs of antibiotics and CP were placed on the nutrient agar plate followed by gentle pressure. These plates were incubated for 24 h at 28°C. The interpretations of results was carried out by measuring the diameter of the inhibition zones (mm) according to Clinical and Laboratory Standards Institute (CLSI) [22] to determine the antibiotic of choice and the best two concentrations of CP.

Experimental Fish

Two hundred and forty apparently healthy Nile tilapia (28.5±0.84g), were procured alive from Al-Abbassa fish farm at Sharkia province, Egypt. The fish were subjected to fourteen days-acclimatization in 60 L glass aquaria (80x 40x30cm) filled with dechlorinated tap water. These aquaria were cleaned by siphoning out all the water, fecal wastes and debris, and then replaced with clean water. Water quality parameters were estimated according to the standard methods [23] and adjusted within the suitable range in all aquaria as following: dissolved oxygen (6.5±0.5mg/l), nitrite (0.04±0.012mg/L), temperature (27±1°C), and ammonia (0.02 ± 0.003mg/L).

Ethical approval and experimental design

The experimental protocol was reviewed and approved by Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC) (Approval number ZU-IACUC/2/F/6/ 2020).

Nile tilapia was distributed randomly into eight groups in triplicates (10 fish /replicate). First group (control) was neither challenged, nor treated. Fish of the second and third groups (CP1 and CP2) were maintained in water containing CP (0.01 and 0.02 g/L, respectively). The fourth group (CE) was exposed to CE at0.5 g/ L. The fifth group (PM) served as a control positive (challenged, non-treated) was intraperitoneally inoculated with pathogenic *P. mirabilis* at a dose of 0.1 mL of 24-h broth (5×10^6 CFU/ mL). The other three groups (CP1-PM, CP2-PM, and CE-PM) were intraperitoneally inoculated with pathogenic *P. mirabilis* then treated with medical bath containing either CP or CE with the same concentrations as previously mentioned.

Clove powder with its both concentrations (CP1 and CP2) as well as CE were added to the water in the second day of the experiment (following the onset of clinical signs in C-PM, CP1-PM, CP2-PM and CE-PM) and continued for 14 days. During this time; the water of each aquarium was exchanged every other

day and replaced with clean, dechlorinated water, then CP1, CP2 or CE was added with the same concentrations as mentioned before. Fish received a balanced, basal diet, at the rate of 3% body weight; they were fed twice daily, at 8.00 am and 4.00 pm throughout the experimental period. The experiment lasted for 15 days, during which all fish were maintained under close inspection, where behavioral changes, clinical signs and mortalities were reported.

Sampling

Sampling was carried out at the end of the treatment period (15 days). At first; fish were euthanized using 250 mg/L of tricaine methanesulfonate (MS-222, Argent laboratories, India), thereafter, nine fish from each group were used for collection of blood samples from the fish caudal blood vessels. Blood samples were collected without anticoagulant then centrifuged at 3000 *xg* for 10 min for serum separation. The serum samples were stored at -20°C till used [24]. Biochemical investigation was carried out to assess stress related parameters, liver and kidney functions, and immunological response. Moreover, three liver specimens from each group were freshly collected for investigation of oxidative stress response.

Serum stress related assay

The serum glucose and cortisol as stress related parameters levels were evaluated by way of spectrophotometry, following protocols reported by Trinder [25] and Burtis and Ashwood [26], respectively.

Analysis of immune state biomarkers

Lysozyme activity was measured using spectrophotometry following the method of Ellis [27]. The levels of nitric oxide (NO) were assayed spectrophotometrically following the protocol of Rajaraman*et al.* [28]. A total amount of 100 µL of serum for each sample was added to the same volume of Griess reagent; the previously mentioned mixture was incubated in a 96 well micro titer plate, for 10 minutes at 27°C. The optical density was measured, spectrophotometrically at 570 nm by ELISA reader.

Assessment of liver and kidney injury markers

The liver alanine (ALT, Ref No.; 1001170) and aspartate (AST, Ref No.; 1001160) aminotransferases and kidney functions as creatinine (Ref No.; 1001115) and urea (Ref No.; 1001323) using the Spin react kits (Esteve De Bas, Girona, Spain); were measured according to Burtis and Ashwood [26], Fossati*et al.* [29] and Kaplan [30] respectively.

Hepatic antioxidant activity

Freshly collected liver samples (three samples/ group) were used to assess the activities of catalase (CAT), superoxide dismutase (SOD), and reduced glutathione content (GSH) content calorimetrically, using the methods described by Aebi [31], McCord and Fridovich [32], and Beutler [33], respectively.

Statistical analysis

Data was analyzed by using SPSS 16.0 software through one-way ANOVA. Tukey's multiple comparisons post hoc test was conducted to compare means among the different treatments groups where the value of $P < 0.05$ was considered as statistically significant. Data were presented as a mean ± *SE*.

Results

Results of antimicrobial susceptibility testing

As depicted in Table 1, cephalexin was the antibiotic of choice for treatment of *O. niloticus* experimentally infected with *P. mirabilis* (the inhibition zone diameter =

20 mm). Moreover, CP was used in the treatment trail in two concentrations; CP1 (0.01 g/L) and CP2 (0.02 g/L).

Table 1. Results of antibiotic sensitivity test of *P. mirabilis.*

Antimicrobial disc	Disc concentration (µg)	Interpretation
Amoxicillin (AM)	25	**R**
Ampicillin (AM)	10	**R**
Azithromycin (AZM)	10	**I**
Cefixim (CFM)	30	**I**
Cefotaxim (CTX)	30	**I**
Cephalexin (CEP)	30	**S**
Ciprofloxacin (CIP)	5	**R**
Enrofloxacin (EN)	10	**R**
Florfenicol (FFC)	30	**R**
Nitrofurantoin (F)	100	**I**
Ofloxacin (OFX)	10	**R**
Streptomycin (S)	10	**R**
Gentamycin (GM)	10	**I**
Oxolinic acid (OA)	2	**I**
Oxytetracycline (OX)	30	**R**
Erythromycin (E)	15	**R**

R: Resistant I: Intermediate S: sensitive

Clinical signs, behavioral alterations and mortalities

Neither mortalities (%) nor clinical symptoms or behavioral alterations were recorded in the control, CP1, CP2, and CE groups. In contrast, among the challenged fish groups; *P. mirabilis*-challenged Nile tilapia that received no treatment (PM group) revealed the highest mortality percentage (85%), followed by CP1-PM (50%), CE-PM (30%) and finally, CP2-PM, which possessed the lowest mortality percentage (20%).

P. mirabilis challenged fish showed behavioral alterations that appeared in the form of anorexia, dullness and lowered escape reflex (decreased response to external stimuli). The most pronounced clinical signs were; ascites, unilateral and/or bi-lateral exophthalmia, loss of scales, and erythematous areas of external structures including base of fins and the lower jaw. Different degrees of fin rot particularly of the caudal fin were also noticed. The post mortem findings revealed congestion of internal organs together with enlarged liver and distended gall bladder.

Following treatment by CP; marked improvement in fish appetite and clinical signs were recorded in a concentration-dependent manner. In addition, CE-treated fish also showed active response

to external stimuli and pronounced alleviation of external lesions.

Effect on serum glucose and cortisol levels

The data presented in Figure 1, showed that, serum values of both glucose and cortisol were significantly declined ($P < 0.05$) in CP2 and CE groups, compared to the control group, meanwhile, their levels in CP1 were not significantly changed. Although, the levels of these stress indicators were markedly elevated in the challenged, non-treated fish (PM) compared to the control one. On contrary, their levels revealed significant reduction ($P< 0.05$) in CP2-PM and CE-PM groups followed by CP1-PM group.

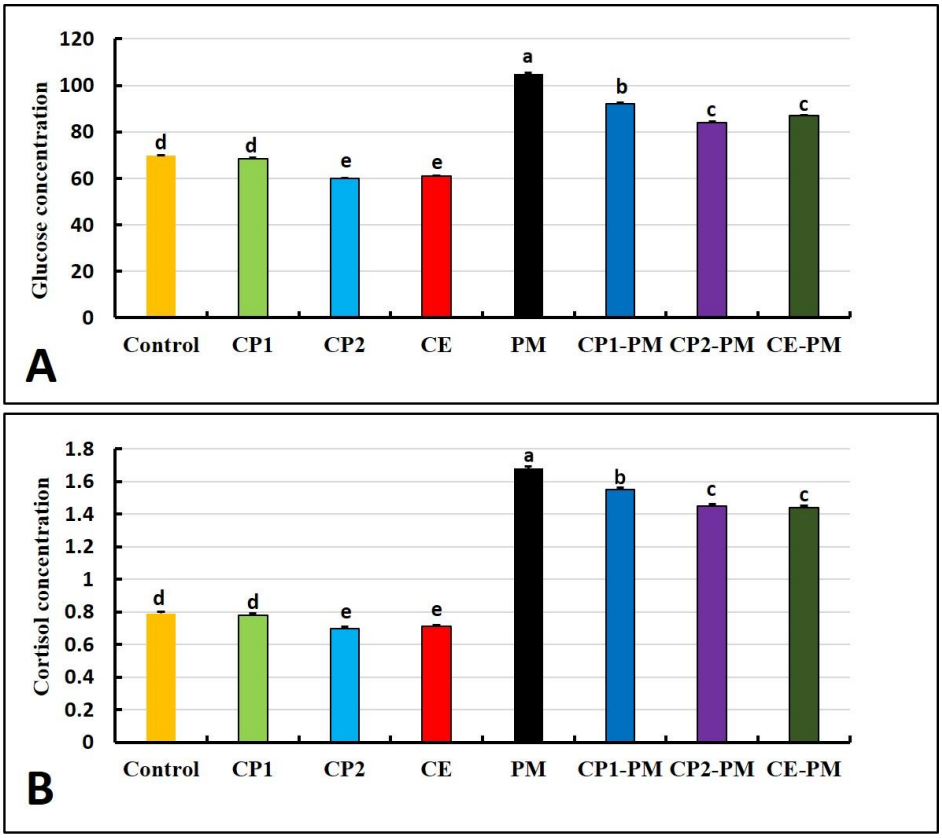

Figure 1: Stress indicators of *O. niloticus* experimentally infected with *P. mirabilis* (PM) and treated with clove powder (CP) and cephalexin (CE) for 15 days. (A) Glucose (mg/dL). (B) Cortisol (µg/dL).Bars with different superscripts are significantly different (one-way ANOVA, $P< 0.05$).

Consequence on immune response biomarkers

As highlighted in Figure2A, serum lysozyme activity was significantly boosted ($P < 0.05$) in CP2 group followed by CP1group, respectively, compared to the control one. However, marked decline in the lysozyme activity was noticed in *P. mirabilis* challenged fish relative to the control one. In contrast, lysozyme activity showed significant rise in CE-PM and CP2-PM followed by CP1-PM compared to the PM group.

The level of NO showed significant increase ($P < 0.05$) in CP2 group followed by CP1 group when compared to that of control group (Figure 2B). On the other hand, its level was significantly lowered

($P < 0.05$) in PM group compared to the control one. In contrast, the treated groups (CP1-PM, CP2-PM, and CE-PM) revealed marked improvement in NO level relative to the PM group, but still exhibited significantly lower levels than that of the control group.

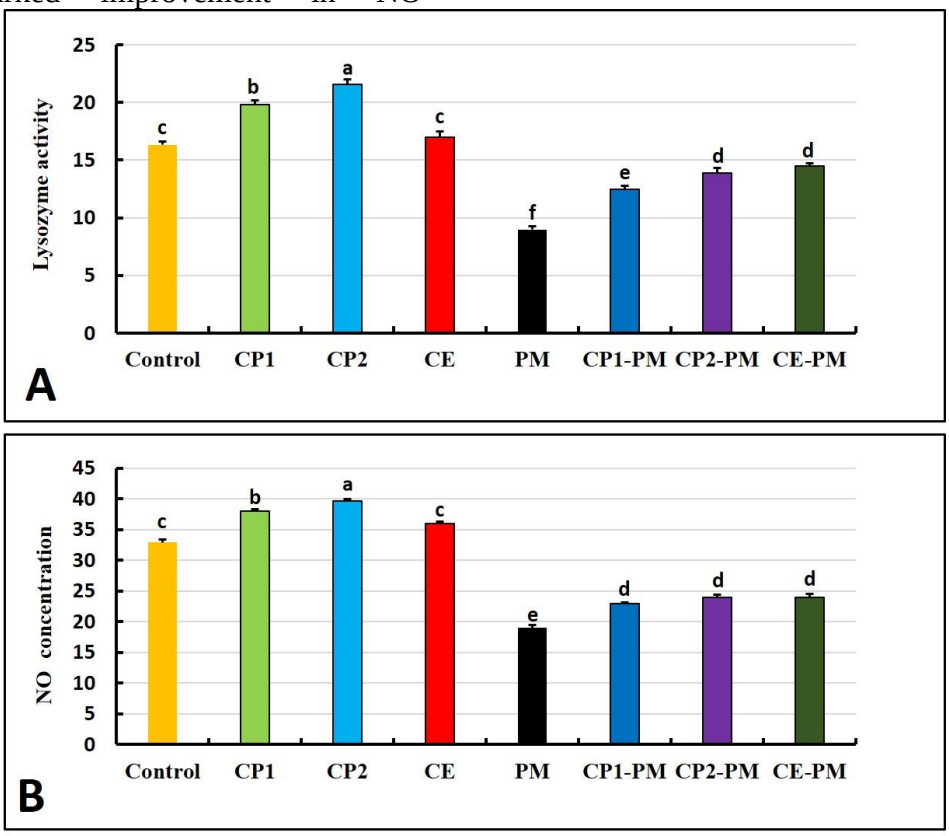

Figure 2: Immunological indicators of *O. niloticus* experimentally infected with *P. mirabilis* (PM) and treated with clove powder (CP) and cephalexin (CE) for 15 days. (A) Lysozyme activity (µg/mL). (B) Nitric oxide (NO; µmol/L). Bars with different superscripts are significantly different (one-way ANOVA, $P< 0.05$).

Impact on liver and kidney function biomarkers

The serum values of hepatic and renal efficiency biomarkers were depicted in Table 2. The levels of both ALT and AST were not significantly varied in CP1, CP2 or CE groups compared to the control group although the highest level of these enzymes was recorded in the PM group as compared with the control one. However, their levels were significantly improved ($P < 0.05$) in CP-treated groups with its both concentrations (CP2-PM and CP1-PM) and also CE-PM group.

Serum levels of renal efficiency biomarkers (urea and creatinine) were also significantly declined ($P < 0.05$) in CP1and CP2 compared to the control group, whereas, their levels was not significantly changed in CE group. Among *P. mirabilis* challenged groups, CP2-PM, CE-PM, and CP1-PM showed marked decreases in these indices; meanwhile PM group exhibited the highest level of these biomarkers relative to the control group.

Table 2. Hepatorenal function indicators of *O. niloticus* experimentally infected with *P. mirabilis* (PM) and treated with clove powder (CP) and cephalexin (CE) for 15 days.

Parameters	ALT (µg /dL)	AST (µg /dL)	Creatinine (mg /dL)	Urea (mg/dL)
Control	14.30 ± 0.07^{d}	19.70 ± 0.05^{d}	0.78 ± 0.005^{d}	21.47 ± 0.75^{d}
CP1	11.56 ± 0.16^{d}	15.86 ± 0.07^{d}	0.55 ± 0.005^{e}	15.46 ± 0.33^{e}
CP2	10.80 ± 0.10^{d}	15.26 ± 0.17^{d}	0.52 ± 0.005^{e}	13.90 ± 0.37^{e}
CE	14.23 ± 0.20^{d}	18.65 ± 0.17^{d}	0.75 ± 0.008^{d}	21.10 ± 0.25^{d}
PM	32.61 ± 0.20^{a}	35.63 ± 0.12^{a}	1.57 ± 0.01^{a}	35.81 ± 0.73^{a}
CP1-PM	26.15 ± 0.09^{bc}	30.88 ± 0.19^{b}	1.26 ± 0.005^{b}	30.82 ± 0.33^{b}
CP2-PM	28.15 ± 0.08^{b}	29.73 ± 0.14^{c}	1.18 ± 0.03^{c}	29.23 ± 0.12^{c}
CE-PM	23.16 ± 0.08^{c}	28.72 ± 0.36^{c}	1.18 ± 0.02^{c}	28.50 ± 0.28^{c}

ALT, alanine aminotransferase; AST, aspartate aminotransferase. Means within the same column carrying different superscripts are significant at $P < 0.05$.

Activities of CAT and SOD enzymes, and GSH content

The data presented in Table 3 clarified that levels of SOD, CAT, and GSH were significantly elevated ($P < 0.05$) in CP2 followed by CP1 then CE groups, respectively, compared to the control group. However, the PM group exhibited the lowest values of these indicators compared to the control. On the other hand, the activity of CAT, SOD, and GSH was significantly enhanced following treatment by either CP (CP2 and CP1) or CE.

Table 3. Hepatic antioxidant indicators of *O. niloticus* experimentally infected with *P. mirabilis* (PM) and treated with clove powder (CP) and cephalexin (CE) for 15 days.

Parameters	CAT (U/g tissue)	SOD (U/g tissue)	GSH (mmol/ g tissue)
Control	14.96 ± 0.39^{d}	5.66 ± 0.04^{d}	1.59 ± 0.05^{c}
CP1	18.53 ± 0.26^{b}	8.33 ± 0.27^{b}	2.92 ± 0.03^{b}
CP2	19.73 ± 0.13^{a}	9.19 ± 0.20^{a}	3.70 ± 0.16^{a}
CE	16.25 ± 0.45^{c}	6.84 ± 0.17^{c}	1.53 ± 0.15^{c}
PM	9.44 ± 0.06^{g}	1.78 ± 0.03^{g}	0.62 ± 0.03^{f}
CP1-PM	11.39 ± 0.24^{f}	3.65 ± 0.12^{f}	0.92 ± 0.01^{e}
CP2-PM	13.03 ± 0.52^{e}	4.19 ± 0.03^{e}	1.06 ± 0.07^{d}
CE-PM	13.83 ± 0.04^{e}	4.77 ± 0.07^{e}	1.06 ± 0.07^{d}

CAT, catalase; SOD, superoxide dismutase; GSH, reduced glutathione content. Means within the same column carrying different superscripts are significant at $P < 0.05$.

Discussion

Bacterial infections are among the most threatening problems facing aquaculture industry all over the world where they are considered the main causes of deaths in cultured fishes [34]. Correspondingly, this investigation was designed to address the pathogenic influence of *P. mirabilis* on health status, immunity and survival rate of *O. niloticus* together with trials for treatment using either CE antibiotic or nutraceuticals (CP) as antibacterial agents.

In the current study, neither clinical signs nor mortalities were recorded in the non-challenged groups, which exposed to water containing CP or CE. In contrast, Nile tilapia challenged with *P. mirabilis*

exhibited high mortality rates and several behavioral alterations and clinical symptoms. These behavioral abnormalities and clinical signs may be attributed to the pathogenic impact of *P. mirabilis* that could be stems from the action of several virulence factors expressed by the bacterium including hemolysin toxin, *PtA* and *zapA* proteases, which exert their proteolytic action via the degradation of both host structural proteins and the immune system, respectively, in addition to the bacterium ability to swarm on the solid surfaces, thus protect itself from the host defense system [19,35]. These findings are in line with those mentioned by Pattanayak*et al.* [9] who documented that; cultured Indian major carp (*Labeo rohita*) exhibited high mortality rates and after through diagnostic steps; *P. mirabilis* infection was approved to be the cause behind these high losses. A similar finding was also obtained by Zhai *et al*. [36] who recorded 100% mortality in yellow catfish and zebrafish challenged by *P. mirabilis* using in the immersion and intraperitoneal route of injection, respectively.

On the other hand, *P. mirabilis* challenged groups that were treated with CP with its both concentrations either the higher (CP2-PM) or the lower one (CP1-PM); showed alleviation of the clinical symptoms with simultaneous reduction of mortalities in a concentration-dependent manner. The lower mortality rates in the clove-treated groups may be returned to the potent antibacterial action of the phenolic constituents of clove mainly eugenol, in addition to their powerful antioxidant activities, which enhances the beneficial impact of the intestinal micro biota, therefore, wall off the hazard effects of the invading systemic bacterium. Our results were consistent with those obtained by Xu*et al.* [37] who approved that essential clove oil revealed powerful antibacterial potency against *Staphylococcus aureus.* Similar findings were also documented by Rattanachaikunsopon and Phumkhachorn [38] who noticed significant reduction in the mortality rate of *Lactococcus garvieae*- challenged Nile tilapia in response to the treatment with clove oil. Concerning the lowered mortalities in response to CE treatment in CE-PM; this could be attributed to the bactericidal action of CE, which stems from the action of the beta-lactam ring that is used to through inhibiting the synthesis of peptidoglycan, which is responsible to mechanical stability of the bacterial cell wall [39].

The non-specific immune system of fish represents the primary weapon for protection against a large diversity of pathogens; it's of a major importance in fish compared with mammals. Lysozymes represent a crucial component of the innate immune response in freshwater fishes [40]. Fish lysozyme not only exerts a lytic action against both Gram-negative and Gram-positive bacteria, but also, It motivates the complement system and phagocytes [41]. Meanwhile, NO not only possesses a potent killing capacity, but also it is capable of deactivating the main enzymes responsible for the cytotoxic reactions managed by macrophages [42]. In the present study, fish challenged with *P. mirabilis* suffered severe impairment of the immunity in the form of significant decline in lysozyme and NO when compared to the control group. These findings may be attributed to the virulence factors of *P. mirabilis* like Zap A protease, which is responsible for degrading a wide variety of structural proteins and immune system factors [43]. Additionally, *P. mirabilis* possesses the *zapA* genes and the gene encoding the PtA protease; that possesses an effective contribution to the produced cytotoxicity and bacterial autoaggregation in kidney and bladder cell lines [44].

Under a stress condition, the fish responds immediately through primary and secondary responses. Thus, the

primary response is mediated by the central nervous system (CNS) through the perception of an altered state and consequently the stress hormones including cortisol are released [45]. Meanwhile, the secondary responses is mediated by the action of the released stress hormones leading to alteration in the hematological and cellular chemistry, such as elevation of the blood glucose level [46]. In the same context, *P. mirabilis*- challenged fish exhibited significantly increased levels of stress indicators (glucose and cortisol). This result was similar to those documented by Ellis*et al.* [47] who reported that the rise of serum cortisol level is a common feature in fish exposed to acute bacterial infection.

In contrast, treatment of *P. mirabilis* – challenged Nile tilapia with either CE antibiotic or CP efficiently modulated the fish immunological response, which was reflected in a marked rise in the immune indices (lysozyme and NO). This was also accompanied with relief of the stress condition, which was indicated by the significant decline in the levels of both glucose and cortisol serum levels. These finding could be due to the powerful role of the therapeutic strategies in combating the bacterial infection and trials to restore the normal state of the fish. Similar findings were supported by Saeed *et al.* [48] who stated that clove oil has strong immuno-stimulatory impact in fish; thus it could substitute antibiotics to fight bacterial fish pathogens. Clove may possess the efficacy to stimulates the useful bacteria as reported by Abdel Rahman*et al.* [19], therefore, improve the utilization of the beneficial components of clove powder that augment the activity of lysozymes and NO; hence enhance the immunological status of Nile tilapia.

In this study, Nile tilapia challenged with *P. mirabilis* (PM) possessed a remarkable rise ($P < 0.05$) in the serum hepato-renal damage biomarkers (ALT, AST, urea and creatinine) relative to the control group and also the treated ones. Such increase of hepatic enzymes may be due to the damage of the cells of both liver and kidney that aroused from the action diversity of genes responsible for the virulence and pathogenicity of *P. mirabilis* secreted by the bacterium. In line with our results; Pattanayak *et al.* [9] observed multiple histopathological lesions in the posterior kidney of *P. mirabilis*- infected *L. rohita*.

On the other hand, CP-treated fish revealed hepato-protective effect that was represented by the reduction in of liver enzymes. These findings were supported by Abdel Rahman *et al.* [19] who attributed this positive impact to the strong antioxidant and antimicrobial activities of clove components.

Liver is the main organ responsible for detoxification in all vertebrates including fish; therefore, it is usually exposed to numerous endogenous and exogenous free radicals which result from the degradation of metabolic products [49]. CAT, GSH and SOD are the crucial antioxidant enzymes, which exhibits a major contribution in indicating the oxidative status in freshwater fish [50, 51]. Both GSH and SOD have been reported to ameliorate the oxidative injury of different cells [52, 53]. The outcomes of this study emphasized that; *P. mirabilis* challenged fish, suffered from significant oxidative distress, which was indicated by the marked decline in the levels of CAT, SOD and GSH. This may be attributed to the micro colonies produced by multiplication of the bacterium as well as biofilm formation, which resulted in an oxidative stress condition represented by decline in antioxidant parameters [54]. On the other hand, treatment of challenged fish with CP resulted in effective relief of the oxidative stress, which was approved by triggering the activity of the antioxidant biomarkers. Hence, we could attribute these findings to hepatic protective effect of the powerful antioxidant enzymatic reactions, which

stem from the potent antioxidant constituents of clove.

Conclusion

Returning to our results, we can conclude that *P. mirabilis* represents a serious threatening bacterial disease of Nile tilapia; it induced abnormalities in fish behavior, severe clinical signs and high mortalities. Infection by *P. mirabilis* was also associated with malfunction of the immune system, oxidative stress, and impairment in hepato-renal function. Nonetheless, the usage of nutraceuticals like CP; enhanced the immune response, anti-oxidant activity and hepato-renal efficiency. Consequently, we highly recommend using CP (0.02 g/L) in form of aqueous additives as a natural, potent antibacterial agent to enhance the resistance of Nile tilapia against *P. mirabilis* challenge.

Acknowledgment

The authors acknowledge members of aquatic animal medicine department, Faculty of Veterinary Medicine, Zagazig University, Egypt for their support and active cooperation.

Conflict of interest

None of the authors have any conflicts of interest to declare.

References

[1] FAO (Food and Agriculture Organization) (2022): The state of world fisheries and aquaculture. Towards blue transformation.

[2] Sampels, S. (2014): Towards a More Sustainable Production of Fish as an Important Protein Source for Human Nutrition. Fisheries Livest Prod, 2: 119.

[3] FAO (Food and Agriculture Organization) (2019): The State of World Fisheries and Aquaculture - Meeting the sustainable development goals. FAO Rome, Italy.

[4] Ahmed, S.A.;Ibrahim, R.E.; Farroh, K., Y.; Moustafa, A. A.; Al-Gabri, N., A.; Alkafafy, M. and Amer, S. A. (2021):Chitosan vitamin E nanocomposite ameliorates the growth, redox, and immune status of Nile tilapia (*Oreochromis niloticus*) reared under different stocking densities. Aquaculture, 541: p. 736804.

[5] Lupatsch, I.; Santos, G.; Schrama, J. and Verreth, J. (2010): Effect of stocking density and feeding level on energy expenditure and stress responsiveness in European sea bass *Dicentrarchus labrax*. Aquaculture, 298: 245–250.

[6]Drzewiecka, D. (2016): Significance and roles of *Proteus* spp. bacteria in natural environments. J Microb Ecol, 72: 741–758.

[7]Saad EL-Deen, A.(2013): Susceptibility rate of Nile tilapia (*Oreochromis niloticus*) and red swamp crayfish (*Procambarus clarkii*) to *Proteus vulgaris* infection. J Assiut Vet Med, 59: 138–145.

[8] Kumar, R.; Swaminathan, T.R.; Kumar, R.G.; Dharmaratnam, A.; Basheer, V. and Jena, J. (2015): Mass mortality in ornamental fish, *Cyprinus carpio* koi caused by a bacterial pathogen, *Proteus hauseri*.Acta Trop, 149: 128–134.

[9] Pattanayak, S.;Kumar, P. R.;Sahoo, M. K.; Paul, A. and Sahoo, P.K. (2018): First field-based evidence of association of *Proteus mirabilis* causing large scale mortality in Indian major carp farming. Aquaculture, 495: 435-442.

[10] Różalski, A.; Torzewska, A.; Moryl, M.; Kwil, I.; Maszewska, A.; Ostrowska, K.; Drzewiecka, D.; Zabłotni, A.; Palusiak, A.; Siwińska, M. and Stączek, P. (2012):

Proteus sp. – an opportunistic bacterial pathogen – classification, swarming growth, clinical significance and virulence factors. Folia Biol Oecol, 8: 1–17.

[11] Rasul, M.G. and Majumdar, B.C. (2017): Abuse of Antibiotics in Aquaculture and it's Effects on Human, Aquatic Animal and Environment. Haya : J Saudi Life Sci,2(3): 81-88.

[12]. Apines-Amar, M.J.S.;Caipang, C., M. A.;Lopez, J. D. M.; Murillo, M. N. A.; Amar, E. C.; Piñosa, L. A.G. and Pedroso, F. L. (2022):*Proteus mirabilis* (MJA 2. 6S) from saline-tolerant tilapia exhibits potent antagonistic activity against Vibrio *spp.,* enhances immunity, controls NH3 levels and improves growth and survival in juvenile giant tiger shrimp, *Penaeus monodon*. Aquac Res, 53 (16): 5510-5520.

[13] Ahmed, S.A.; Reda, R.M. and ElHady, M. (2020): Immunomodulation by *Coriandrum sativum* seeds (Coriander) and its ameliorative effect on lead-induced immunotoxicity in Nile tilapia (*Oreochromis niloticus* L.). Aquac Res, 51(3): 1077-1088.

[14] Mahboub, H.H.,; Faggio, C.;Hendam, B. M.; Algharib, S.A.; Alkafafy, M.; Abo Hashem, M.; Mahmoud, Y. K.; Khamis, T.; Abdel-Ghany, H. M.; Masoud, S. R. and Abdel Rahman, A., N. (2022): Immune-antioxidant trait, *Aeromonas veronii* resistance, growth, intestinal architecture, and splenic cytokines expression of *Cyprinus carpio* fed Prunus armeniaca kernel-enriched diets.Fish Shellfish Immunol, 124: 182-191.

[15] Mahboub, H.H.; Elsheshtawy, H., M.; Sheraiba, N. I.; Fahmy, E. M.; Mohamed, E. A.A.; Abdelnaeim, N. S.; Mohamed, D.; I smail, T., A. and Ahmed, S. A.A. (2022): Dietary black cumin (*Nigella sativa*) improved hemato-biochemical, oxidative stress, gene expression, and immunological response of Nile tilapia (*Oreochromis niloticus*) infected by *Burkholderia cepacia*. Aquac Rep, 22: 100943.

[16] Cortés-Rojas, D.F.; de Souza, C.R.F. and Oliveira, W.P. (2014): Clove (*Syzygium aromaticum*): a precious spice. Asian Pac J Trop Biomed, 4(2): 90-96.

[17] Mittal, M.;Gupta, N.;Parashar, P.; Mehra, V. and Khatri, M. (2014): Phytochemical evaluation and pharmacological activity of Syzygium aromaticum: a comprehensive review. PharmPharm Sci, 6(8): 67-72.

[18] Abdel-Razek, N.; Awad, S.M. and Abdel-Tawwab, M. (2019): Effect of dietary purslane (*Portulaca oleracea* L.) leaves powder on growth, immunostimulation, and protection of Nile tilapia, against *Aeromonas hydrophila* infection. Fish Physiol Biochem, 45(6): 1907-1917.

[19] Abdel Rahman, A.; El-Bouhy, Z.; Wahbah, M. and Ahmed, S.A.A. (2020): Effects of dietary turmeric and clove powder on growth and immune response of the Nile tilapia. Egypt. Aquat Biol Fish, 24(5): 589-608.

[20] Blair, J.; Lenette, E. and Truant, I. (1970): Manual of Clinical Microbiology. American Society for Microbiology. Baltimore, Maryland and Wilkins Company, Washington DC.

[21]Tshabalala, R.; Kabelinde, A.; Kaptchouang Tchatchouang, C.D; Ateba, C.N.; Manganyi, M.C. (2021): Effect of Clove (*Syzygium aromaticum*) spice as microbial inhibitor of resistant bacteria and Organoleptic Quality of meat. J Saudi J Biol Sci, 28(7):3855-3863.

[22] CLSI (2017): Clinical and Laboratory Standards Institute Performance standards for antimicrobial susceptibility testing. Wayne, PA.

[23] APAH (1998): American Water Works Association and Water Pollution Control Pollution Federation. In Franson MAH (ed) Standard methods for examination of water and wastewater, 16th edn. American Public Health Association, Wasington.

[24] Abdel Rahman, A.; Hassanin, M. and ElHady, M. (2019): Growth performance, haematology and intestinal histo-morphology of Nile tilapia fed on Indian Lotus (*Nelumbo nucifera Gaertn*.) leaf powder at different concentrations. Aquac Res,50, 3211–3222.

[25] Trinder, P. (1969): Serum glucose determination. J Ann. Biochem, 6: p. 24.

[26] Burtis, C.A. and Ashwood, E.R. (1994): Tietz textbook of clinical chemistry. Amer Assn for Clinical Chemistry.

[27] Ellis, A.i. (1990): Lysozyme assays. Techniques Fish Immunol, 1: 101-103.

[28] Rajaraman, V.; Nonnecke, B.J.; Franklin, , S. T.; Hammell, D.C. and Horst, R. (1998): Effect of Vitamins A and E on Nitric Oxide Production by Blood Mononuclear Leukocytes from Neonatal Calves Fed Milk Replacer1, 2, 3. J Dairy Sci,81(12): 3278-3285.

[29] Fossati, P.; Prencipe, L. and Berti, G. (1983): Enzymic creatinine assay: a new colorimetric method based on hydrogen peroxide measurement. J Clin Chem, 29(8): 1494-1496.

[30] Kaplan, A. (1984): Clinical Chemistry; The CV Mosby Co.: Toronto, ON, Canada, pp. 418, 437 and 1257–1260.

[31] Aebi, H. (1984): Catalase in vitro, in Methods in enzymology. Elsevier. p. 121-126.

[32] McCord, J.M. and Fridovich, I. (1969): Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J Biol Chem,244(22): 6049-6055.

[33] Beutler, E. (1963): Improved method for the determination of blood glutathione.lab clin Med, 61: 882-888.

[34]Pathirana,H.K.N.S.;Wimalasena,S.H.M.P.; De Silva,B.C.J.; Hossain, S. and Heo, G. (2019): Antibacterial activity of clove essential oil and eugenol against fish pathogenic bacteria isolated from cultured olive flounder (*Paralichthys olivaceus*). Slov Vet Res, 56: 31-38.

[35] Filipiak, A.;Chrapek, M.;Literacka, E.;Wawszczak, M.; Głuszek, S.; Majchrzak, M.; Wróbel, G.;Łysek-Gładysińska, M.;Gniadkowski, M. and Adamus-Białek, W. (2020): Pathogenic factors correlate with antimicrobial resistance among clinical *Proteus mirabilis* strains. Front Microbiol, 11: 579389.

[36] Zhai, W.; Wang, Q.; Zhu, X.; Jia, X. and Chen, L. (2023): Pathogenic infection and microbial composition of yellow catfish (*Pelteobagrus fulvidraco*) challenged by *Aeromonas veronii* and *Proteus mirabilis*. Aquac Fish, 8(2): 166-173.

[37] Xu, J.-G.;Liu, T.;Hu, Q. and, Cao, X. (2016): Chemical composition, antibacterial properties and mechanism of action of essential oil from clove buds against *Staphylococcus aureus*. Molecules, 21(9): 1194.

[38] Rattanachaikunsopon, P. and Phumkhachorn, P. (2010): Potential of

cinnamon (*Cinnamomum verum*) oil to control *Streptococcus iniae* infection in tilapia (*Oreochromis niloticus*). Fish Sci, 76(2): 287-293.

[39] Herman, T.F. and Hashmi, M.F. (2022): Cephalexin, in StatPearls [Internet]. StatPearls Publishing.

[40] Valipour, A.;Heidari, B.; Hadavi, M. and Yousefi, A. (2018): Changes in immune parameters (lysozyme, IgM, C3) in early life stages and broodstock of Siberian sturgeon, *Acipenser baerii*. J FishAquat Life, 26(1): 21-30.

[41] Saurabh, S. and Sahoo, P. (2008): Lysozyme: an important defence molecule of fish innate immune system. Aquac Res, 39(3): 223-239.

[42] Grayfer, L., Kerimoglu, B., Yaparla, A., Hodgkinson, J., Xie, J. and Belosevic, M. (2018): Mechanisms of fish macrophage antimicrobial immunity. Front immunol, 9: 1105.

[43] Belas, R.; Manos, J. and Suvanasuthi, R. (2004): *Proteus mirabilis* ZapA metalloprotease degrades a broad spectrum of substrates, including antimicrobial peptides. Infect Immun, **72**(9): 5159-5167.

[44] Sanches, M.S.;Baptista, A.A. S.;de Souza, M.;Menck-Costa, M. F.; Justino, L.; Nishio, E.K.; Oba, A.; Bracarense, A. P. F.R.L and Rocha, S. P. D. (2020): Proteus mirabilis causing cellulitis in broiler chickens.Braz J Microbiol, 51(3): 1353-1362.

[45] Barton, B.A. and Iwama, G.K. (1991): Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu Rev Fish Dis, 1: 3-26.

[46] Ray, S. and Sinha, R.(2014): Serum cortisol and glucose: Reliable bioindicators of stress in the fish Labeo rohita. J Innov, 1: 6-17.

[47] Ellis, T.;Bagwell, N.; Pond, M., Baynes, S. and Scott, A. P. (2007): Acute viral and bacterial infections elevate water cortisol concentrations in fish tanks. Aquaculture, **272**(1-4): p. 707-716.

[48] Saeed, M.;Khan, M. S.;Younas, U.; Siddique, F.; Niaz, K.; Farooq, Z.; Farag, M. R.; Elnesr, S., S.; Alagawany, M. and Ramadan, M., F. (2022): Clove (*Syzygium aromaticum*) and its derivatives in fish feed, in Clove (*Syzygium Aromaticum*). Elsevier.Inc., p. 257-266.

[49] Cong, B.; Liu, C.; Wang, L. and Chai, Y. (2020): The impact on antioxidant enzyme activity and related gene expression following adult zebrafish (*Danio rerio*) exposure to dimethyl phthalate.Anim,10(4): 717.

[50] Wu, P., Liu, Y., Jiang, W.; Jiang, J.; Zhao, J.; Zhang, Y.; Zhou, X. and Feng, L. (2017): A comparative study on antioxidant system in fish hepatopancreas and intestine affected by choline deficiency: different change patterns of varied antioxidant enzyme genes and Nrf2 signaling factors.PLoS One, 12(1): e0169888.

[51] Abdel Rahman, A.N.; Maricchiolo, G.; Abd El-Fattah, A.H.; Alagawany, M. and Reda, R.M. (2021): Use of rice protein concentrates in *Oreochromis niloticus* diets and its effect on growth, intestinal morphology, biochemical indices and ghrelin gene expression. Aquac. Nutr 27, 2267–2278.

[52]Alkaladi A.(2019): Vitamins E and C ameliorate the oxidative stresses induced by zinc oxide nanoparticles on liver and gills of *Oreochromis niloticus*. Saudi J Biol Sci, 26(2):357-362.

[53] Ahmed,S.A.A.; Nada, H.S.; Elsheshtawy, H.M.; Ibrahim, S.M.; Fahmy, E.M.;

Khedr, M.H.E.; Moustafa, S.M.; Ismail, T.A.; Gesriha, S.; Assayed, M.E. and Heba H. Mahboub, H.H. (2022): Comparative antitoxic potency of honey and natamycin-supplemented diets against aflatoxicosis and their influences on growth, serum biochemistry, immunohistochemistry, and residual deposition in Nile tilapia (*Oreochromis niloticus*). Aquaculture, 551: p.737934.

[54] Lazãr, V. and Chifiriuc, M.C. (2010): Architecture and physiology of microbial biofilms. Roum Arch Microbiol Immunol 69(2): 95-107.

الملخص العربي

التأثيرات المناعية والمضادة للأكسدة والمضادة للبكتيريا لمسحوق القرنفل في البلطي النيلي المصاب معمليا ببكتيريا البروتيوسميرابيليز دراسة مقارنة مع المضاد الحيوي السيفالكسين

زينب البوهي[1], عفاف نورالدين عبدالرحمن[1], محمد وهبه[2], شيماء عبدالكريم علي أحمد[1]
[1]قسم طب الاحياء المائية, كلية الطب البيطري, جامعة الزقازيق, ص.ب. 44511, الزقازيق, الشرقية, جمهورية مصر العربية.
[2] الادارة البيطرية, اجا, محافظة الدقهلية.

في الوقت الحالي ، أصبح العلاج بالأعشاب بديلاً هامًا يستخدم على نطاق واسع في تربية الأحياء المائية وذلك للحد من إستخدام المضادات الحيوية والمواد الكيميائية الأخرى أثناء عملية إنتاج الأسماك. لقد أجريت الدراسة الحالية لتقييم الفعالية العلاجية لمسحوق القرنفل كحمام مائي علاجي مقارنة بالمضاد الحيوي السيفالكسين وذلك في علاج البلطي النيلي الذي تم حقنه معمليا ببكتيريا البروتيوس ميرابيليز .تم تقسيم الأسماك (العدد=240) إلى ثماني مجموعات ، كل مجموعة بها 3 مكررات. المجموعة الأولى (المجموعة الضابطة) وهي الأسماك التي لم يتم حقنها بالبكتيريا أو معالجتها. بالنسبة لأسماك المجموعتين الثانية والثالثة فقد تم تربيتها في الماء المحتوي على مسحوق القرنفل بتركيز 0.01 و 0.02 جم /لتر علي التوالي. المجموعة الرابعة تم تربيتها في ماء يحتوي علي السيفالكسين بتركيز0.5 جم/لتر. المجموعة الخامسة ؛ كانت بمثابة المجموعة الضابطة الإيجابية حيث تم حقنها معمليا ببكتيريا البروتيوس ميرابيليز ولم تعالج. وفي الوقت نفسه ، فقد تم معالجة المجموعات الثلاث الأخرى بنفس البكتيريا ، ثم عولجت بحمام علاجي يحتوي إما على مسحوق القرنفل أو السيفالكسين بنفس التركيزات كما ذكرنا سابقًا. إستمرت التجربة لمدة 15 يومًا ، تم خلالها إبقاء جميع الأسماك تحت الملاحظة.

وقد أظهرت النتائج أن الاسماك التي تم حقنها معملياً بالبكتيريا ولم تعالج (المجموعة الضابطة الإيجابية), قد سجلت أعلى معدل نفوق (85٪) ، وأعراض سريرية ملحوظة ، بالإضافة إلي إرتفاع مستوي مؤشرات الإجهاد في السيرم (الجلوكوز والكورتيزول) وكذلك مؤشرات وظائف الكبد و الكلى. وقد لوحظ إنخفاض ملحوظ في مؤشرات الإستجابة المناعية (الليزوزيم وأكسيد النيتريك) ومضادات الأكسدة في نفس المجموعة.علي النقيض من ذلك وبشكل مثير للدهشة ، لوحظ تحسن كبير في جميع هذه المؤشرات مع إنخفاض معدل النفوق في المجموعات المصابة التي تم معالجتها بحمام علاجي يحتوي علي مسحوق القرنفل بتركيز 0.02 جم /لتر او السيفالكسين0.5 جم/لتر (20% و 30% علي التوالي). وبالتالي ، فإننا نوصي بإستخدام مسحوق القرنفل بجرعة 0.02 مجم/ لتر كعامل طبيعي ومنبه مناعي قوي وأيضا مضاد للبكتيريا بدلاً من المضادات الحيوية لتجنب آثارها السلبية وتمهيد الطريق نحو صناعة تربية الأحياء المائية المستدامة.