Evaluation of the anti-bacterial activity of phytol against *Erysipelothrix piscisicarius* infection in Nile tilapia (*Oreochromis niloticus*)

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Abstract

About 50% of fisheries are now supported by aquaculture, one of the world's fastest expanding food producing sectors. While there has been a clear decline in catch fisheries, there has been an increase in the global demand for fish, making it a sustainable business and top priority in the world. Aquaculture disease outbreaks, particularly those caused by bacteria like *Erysipelothrix piscisicarius*, are a significant barrier to profitability. Due to the high expense of antibiotics, the limited duration of protection they provided, the necessity for repeated treatments during prolonged disease outbreaks, the challenges posed by resistant strains, and the rising levels of hazardous residues in carcasses, the use of antibiotics has been discouraged. As an alternative, phytochemicals like phytol can be employed. This investigation looked at the haematology, biochemistry, immunological profiles, antioxidants, histopathology, and lifespan of Phytol-gavaged Nile tilapia, *Oreochromis niloticus*, which had been infected with *E. pisciscicarius*. *E. pisciscicarius* (1.4 x10 CFU/mL/g) was intraperitoneally injected into juvenile Nile tilapia (n=120, mean weight= 4.22g) before being divided among 12 1m^3 tanks. Ten fish were housed in each tank. The fish were gavaged with phytol solution at 0, 1.75, 3.50, or 7.00 mg/g after 24 hours of infection, and 14 days' worth of clinical changes were monitored. The outcomes demonstrated that for the first three days after infection, the fish's body did not physically change. However, on Day 4, cracks were seen on the fish's head region in both the Control and 1.75 mg/g, and on Day 10, weak traces of blood surrounding the operculum of the fish grew more obvious. The fish gavaged with 2.50 mg/g phytol experienced the highest survival rate (60.0%), while the control group experienced the lowest (20.0%). With their lowest values found in the control group, significant differences were found in PCV, Haemoglobin, Heterocytes, RBC, WBC, Platelets, Lymphocytes, MCH, MCHC, and Hetero:Lymphocyte in fish (P 0.05). Fish gavaged with phytol had significantly higher superoxide dismutase, catalase, lysozyme, and respiratory burst activity than the control. The control group showed cryptal and surface enterocyte necrosis, intestinal villi atrophy, and gill lamellae hyperplasia, while the treated group, particularly at doses of 3.50 to 7.00 mg/g phytol, showed only mild to no lesions. The study came to the conclusion that *Erysipelothrix pisciscicarius* infection has a significant impact on Nile tilapia survival, haematology, antioxidants, and immunity. At the recommended level of 3.21 mg/g, phytol can also be used to treat and control the *Erysipelothrix pisciscicarius* infection in Nile tilapia.

Keywords: *Erysipelothrix pisciscicarius*, Nile tilapia, Haematology, Antioxidant, Immunity, Survival

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Introduction

Aquaculture is one of the world's fastest expanding food production sectors, accounting for over half of all fisheries products (FAO, 2010). Aquaculture has developed into a multibillion dollar business, according to FAO (2006). Aquaculture is becoming a business and occupying a priority list in the world, remedying the noticeable decrease in capture fisheries but there are increases in the demand of fish throughout the world (Adeshina and Umma, 2012).

Fish is the cheapest form of animal protein taken by the average Nigerian, accounting for approximately 40% of total protein intake (Atanda, 2007). One-fifth of all animal protein consumed worldwide comes from fish, which has increased fivefold over the past 40 years from 20-98 million metric tonnes in 1993 to over 150 million metric tonnes by the year 2010. (Olagunju *et al*., 2007). One of the first ever cultured species of fish is the Nile tilapia, *Oreochromis niloticus*.

Disease outbreaks in cultured fish are a major impediment to profitability. Before 2014, *Erysipelothrix* species have occurred in aquaculture farms. Results of this and other studies revealed a novel emerging fish pathogen known as *E. piscisicarius* (Pormanski *et al*., 2019). The introduction of specific-pathogen-free brood stocks, use of sanitary certificates with quarantine measures, optimization of feed, improvement of water quality, adequate sanitation together with effective health management are the primary preventative strategies that control bacterial illnesses in fish farms (FAO, 2008).

The demand for utilizing large amounts of antibiotics is rising along with the expansion and intensification of fish farms (Bruun *et al*., 2000). It is advised against heavily relying on antibiotics to combat infectious and contagious diseases in fish farms because DOIng so has a number of drawbacks, including the cost of antibiotics, their limited duration of protection, the need for repeated treatments during prolonged outbreaks of disease, the challenges posed by resistant strains, and the increased presence of harmful residues in carcasses (Miranda and Zemelman, 2002). As an alternative, phytochemicals like Phytol can be employed. Fruits, vegetables, legumes, grains, and plant-based beverages like tea and wine all include phytochemicals, a vast class of plant-derived molecules (Arts and Hollman, 2005). Numerous activities, including growth, feed consumption, immune stimulation, anti-stress, and promotion of fish's antibacterial characteristics, have been reported to be improved by phytochemicals (Citarasu, 2010; Chakraborty and Hancz, 2011). Exploiting plants, plant extracts, or natural plant components as viable all-natural options for raising fish productivity has received a lot of attention. The *Erysipelothrix pisciscicarius* infection cause huge mortality in fish and should be controlled for profitable business. The search for an alternative to antibiotics such as phytol cannot be

overemphasized. This is because antibiotics have been discouraged due to the residual effects and environmental degradation, hence the need for this study. This study evaluated the anti-bacterial activity of phytol against *Erysipelothrix piscisicarius* infection in Nile tilapia (*Oreochromis niloticus).*

Materials and methods

Collection of Erysipelothrix piscisciarius

The bacteria isolate was collected from Faculty of Veterinary Pathology, University of Ibadan, Nigeria and prepared in nutrient agar broth at 1.4 x10 CFU/mL and used immediately.

Collection of Nile Tilapia and Fish Culture

Oreochromis niloticus juvenile (n=120, mean weight=4.22g) fish was collected and transported in oxygen-bag from from Offa, Kwara state. Before the experiment, the fish were acclimated in rectangular plastic tanks (46.5 x 23.5 x 32 cm) and fed twice daily for seven days. In a completely random design, the fish were distributed to 12 tanks, each holding 10 fish in triplicate. On a regular basis, tap water from a water storage tank was entirely drained out of the experimental tanks and replaced with fish feaces-contaminated water. The Fish were infected on the 8th day after acclimatization at about 12:30pm.

Introduction of Erysipelothrix pisciscicaius

The bacteria isolates (*E. piscisiciarius*) at 1.4 x10 CFU/mL/g was intraperitoneally injected to fish (120 fish at 10/tank) juveniles and observed for clinical changes for 14 days.

Administration of Phytol

The phyto was administered to the fish 24 hours post infection) through gavaging at 0, 1.25, 3.50, 7.00 mg/g observed for 14 days.

Hematological and biochemical assays

Five fish were fasted for 24 hours prior to blood sample at the end of the experiment. With buffered tricaine methane sulfonate (30 mg/L), fish were put to sleep. The caudal vein was used to collect blood samples, which were then delicately transferred into lithium-heparinized tubes at room temperature. Two sets of Eppendorf tubes were used to separate the collected blood. For hematology (counting of hemoglobin, hematocrit, platelets, red blood cells (RBC), and white blood cells (WBC), one set of samples contained sodium heparinate (20 U/L), whereas the second set was left without anticoagulant to clot at 4°C and was centrifuged at 5000 rpm for 10 min at room temperature to obtain a serum. After diluting the blood with phosphate-buffered saline, RBCs were counted using a Neubauer hemocytometer under a light microscope (pH 7.2). Fresh blood was placed in glass capillary tubes, centrifuged for 10 minutes in a microhematocrit centrifuge, and the packed cell volume was measured to calculate the hematocrit (Ht) values immediately after sampling. According to Vankampen and Ziglstra, colorimetric measurements of

cyanmethemoglobin production were used to determine the amounts of hemoglobin (Hb) (1961). The Brown methods were used to calculate the RBC and WBC numbers (1980). Blood films were stained with the Wright-Giemsa stain after being fixed in methanol to show the differential count of lymphocytes, heterocytes, monocytes, eosinophils, and basophils.

Antioxidant and innate immunity assays

Using diagnostic reagent kits (Randox® Laboratories, Crumlin, County Antrim, UK), the antioxidant and immune activities in fish serum were assessed. The ferricytochrome -C technique was used to test the superoxide dismutase (SOD) activity with xanthine/xanthine oxidase serving as the source of superoxide radicals. The reaction mixture contained 0.1 mM ethylenediaminetetraacetic acid, 0.1 mM xanthine, 0.013 mM cytochrome C, and 0.024 IU/mL xanthine oxidase in addition to 50 mM potassium phosphate buffer (pH 7.8). The amount of enzyme required to achieve a 50% inhibition of the ferricytochrome-C reduction rate, measured at 550 nm, was defined as one activity unit (McCord and Fridovich, 1969). According to Aebi, the decrease in H2O2 concentration at 240 nm was used to measure the catalase (CAT) activity (1984). The reaction mixture included 10.6 mM H2O2 and freshly made 50 mm potassium phosphate buffer (pH 7.0). Fish sera's lysozyme activity was measured using the lysoplate method (Grinde, 1989). In a nutshell, 0.60 mg/mL Micrococcus luteus was cast in 1% agarose gel with 50 mM phosphate buffer (Difco BD Co, Franklin Lakes, NJ) (pH 6.2). 25 L of serum samples were placed in wells (6 mm) made in nutrient agar plates, which were then incubated at 25°C for 20 hours. A standard curve made with lysozyme from chicken egg white served as the basis for calculating the lysozyme activity. According to Chiu, Guu, Liu, Pan, and Cheng's descriptions, diagnostic reagent kits (Randox, London, UK) were used to quantify respiratory burst activity (RBA) (2007). According to Secombes (1990), with some modifications, RBA of the whole blood was measured using the nitroblue tetrazolium (NBT) assay, which gauges the number of intracellular oxidative free radicals. In a 96-well microtiter plate, 100 mL of the blood suspension was quickly added to each well (Nalge-Nunc, Hereford, UK). For two hours, the plate was kept at 25°C to facilitate cell adhesion. Utilizing brand-new L-15 media, detached cells were rinsed off three times. NBT (1 mg/mL) and phorbol 12-myristate 13-acetate (1 mg/mL) from Sigma-Aldrich were then added to the L-15 medium, and 100 mL was added to each well of the microtiter plate. The microtiter plate was then incubated at room temperature for 1 hour. The supernatant was removed from the plate after incubation, and the NBT reduction was fixed with 100% methanol for 10 minutes. Following a 70% methanol wash, the plate was allowed to air dry. To dissolve the

resultant formazan blue crystals, 140 mL DMSO and 120 mL of 2 M potassium hydroxide were used. RBA was expressed as an NBT reduction, which was evaluated using a microplate reader (Optica, Mikura Ltd., UK) at 630 nm.

Statistical analysis

The data obtained were analyzed and presented as mean±standard deviation with aid of IBM Statistical Package for Social Science version 20. The haematological and antioxidant data was analyzed using one- way analysis of variance (ANOVA). Means were separated using Duncan test at $p<0.05$ significant levels.

Results

Table 1 reveals physical observations of *Oreochromis nilloticus* infected with *Erysipelothrix pisciscicarius* and treated with Phytol.

Table 1: Physical Observations of *Oreochromis nillotics* infected with *Erysipelothrix pisciscicarius* and treated with Phytol.

Traatments	Day 1	Day 4	Day 7	Day 10
Control Treament with *Erysipelothrix pisciscarius*	No physical change observed on the body of the fish	Faint traces of Blood on the operculum of the fish	Blood on the operculum of the fish became more visible	The blood around the operculum of the fish became more visible
Treatment with 25% Phytol	No Physical change observed	No Physical change observed	Yellow pigment on the operculum of the fish	The skin of the fish became pale. Yellow pigment on the operculum of fish has spread to the belly region
Treatment with 50% phytol	No Physical change observed	Faint traces of Cracks on the head region of the fish	Cracks on the head fish became more visible	Yellow pigment on the operculum of the fish
Treatment with 100% phytol	No Physical change observed	No Physical change observed	Yellow pigment on the operculum of the fish	Yellow pigment on the operculum of the fish has spread to the part of the belly region

There was no physical change in the body of the fish post infection for the first 3 days. However on Day 4, faint traces of blood on the operculum of the fish and cracks on the head region of the fish was observed in the Control and 50% Phytol treatment respectively, while the 25% and 100% Phytol treatments still had no Physical change on the body of the fish. Also on day 7, physical changes were observed in the control group of the fish with faint traces of blood on the operculum of the fish which become more visible in Day 10. In the group with Phytol, a Yellow pigment on the operculum was observed in the treatment with 25% and 100% Phytol and this continued till Day 10. The treatment with the dose of 50% Phytol had a slight variation in the

physical changes as the cracks in the head region of the fish became more pronounced in Day 7 and a faint yellow pigment was observed on the operculum region of the fish in Day 10.

Figure 1 actively shows the activity that was observed in the intestine and gills of *Oreochromis niloticus* infected with *Erysipelothrix pisciscicarius* and treated with different doses of Phytol. Necrosios of cryptal and surface enterocytes and villi atrophy of the intestine was observed in the experimental control and the treatments dosed with 100% and 50% Phytol respectively. However, in the treatment dosed with 25% Phytol, there was no observable lesion. The lamellae hyperplasia in the gills was observed in marked, mild and moderate measures in the Control, 100% Phytol, 50% Phytol treatments respectively. The treatment with 25% Phytol however, had no observable lesion.

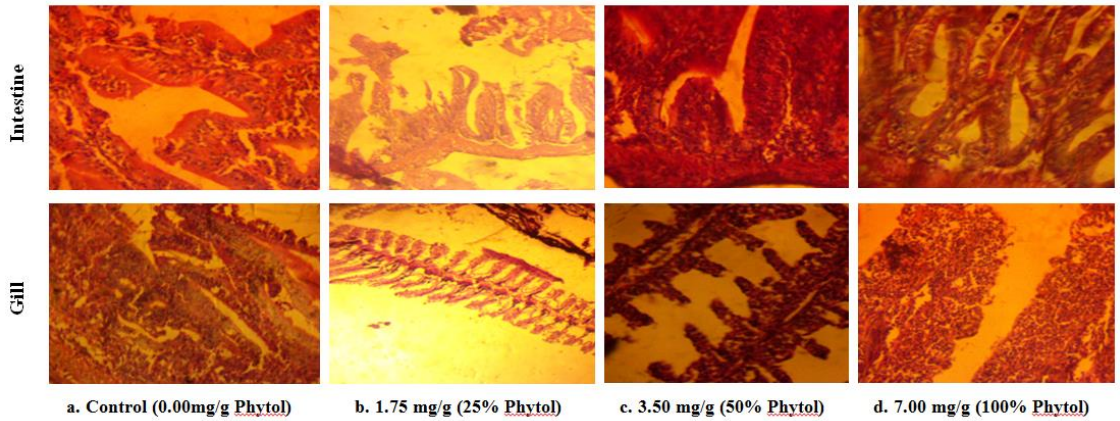

Figure 1: Hispatological result of the gills and intestine of *Oreochromis nilotics* infected with *Erysipelothrix pisciscicarius* and treated with Phyol.

Table 2 reveals the rate of survival of *Oreochromis nilotics* infected with *Erysipelothrix pisciscicarius* and treated with different does of Phytol. The total number of infec26.ted fish in each treatment was 30 and the survival rate of *Oreochromis niloticus* in the different treatments were observed to be 30%, 36.67%, 20% and 26.67% for the Control, 25%Phytol, 50%Phytol, 100%Phytol respectively. The treatment with 25%Phytol had the highest survival rate with 50%Phytol treatment with the lowest.

Table 3 depicts the haematological profiles of *Oreochromis niloticus* infected with *Erysipelothrix pisciscicarius* and treated with different doses of Phytol. Significant difference were observed in PCV, Haemoglobin, Heterocytes, RBC, WBC, Platelets, Lymphocytes, MCH, MCHC and Hetero:Lymphocyte (p<0.05). However, no significant difference were observed in Monocytes, Eosinophilis, Basophil and MCV (p>0.05). Monocytes of treatment 50% phytol had the highest concentration while the lowest was observed in 25%Phytol. Eosinophil and

Basophil have their highest concentrations in treatment of 25%Phytol and their lowest in control treatment and 50%Phytol treatment in case of Basophil. Mean Cell Volume (MCV) has their highest and lowest concentration at 25%Phytol and 50% Phytol, respectively.

Table 2: Survival Rate of Oreochromis niloticus infected with Erysiplothrix piscisicarius and treated with Phytol.

Parameters	Phytol levels			
	Control (0.00mg/g)	1.75 mg/g (25%)	3.50 mg/g (50%)	7.00 mg/g (100%)
No. Infected Fish	30	30	30	30
No. Survived	9	11	6	8
Survival rate (%)	30.00	36.67	20.00	26.67

Table 3: Haematological Index of *Oreochromis niloticus* infected with *Erysipelothrix piscsicarius* and treated with Phytol.

Parameters	Phytol levels			
	Control (0.00mg/g)	1.75mg/g (25%)	3.50mg/g (50%)	7.00mg/g (100%)
PCV (%)	16.67 ± 2.08^{a}	17.33 ± 1.53^{a}	14.00 ± 1.00^{b}	17.33 ± 1.53^{a}
Haemoglobin (g/dL)	6.30 ± 0.20^{a}	5.33 ± 0.78^{b}	4.63 ± 0.25^{b}	5.60 ± 0.70^{a}
Red blood cell ($x10^{6)}$	1.13 ± 0.70^{a}	1.15 ± 0.20^{a}	1.03 ± 0.03^{b}	1.18 ± 0.07^{a}
White blood cell ($x10^{3}$/uL)	17.63 ± 0.83^{a}	17.66 ± 0.30^{a}	14.22 ± 0.03^{b}	15.58 ± 2.54^{ab}
Platelets (x m/ uL)	38.00 ± 8.66^{c}	88.00 ± 2.00^{a}	56.00 ± 1.00^{b}	49.00 ± 2.65^{b}
Lymphocytes (%)	53.00 ± 7.55^{b}	66.67 ± 2.08^{a}	71.00 ± 2.00^{a}	50.67 ± 4.51^{b}
Heterocytes (%)	41.33 ± 8.33^{a}	25.33 ± 1.53^{b}	21.33 ± 2.08^{b}	41.67 ± 6.03^{a}
Monocytes (%)	3.00 ± 1.00^{a}	2.67 ± 0.58^{a}	3.67 ± 0.58^{a}	3.00 ± 1.00^{a}
Eosinophilis (%)	2.33 ± 0.58^{a}	4.33 ± 1.15^{a}	3.67 ± 0.58^{a}	4.00 ± 1.73^{a}
Basophil (%)	0.33 ± 0.58^{a}	1.00 ± 0.00^{a}	0.33 ± 0.58^{a}	0.67 ± 1.15^{a}
Mean Cell Volume (FI)	148.17 ± 24.13^{a}	150.70 ± 12.71^{a}	136.26 ± 6.44^{a}	146.94 ± 9.43^{a}
Mean Cell Haemoglobin (pg)	55.82 ± 1.86^{a}	41.45 ± 1.29^{b}	45.11 ± 1.39^{b}	47.93 ± 8.78^{a}
MCHC (pg)	38.25 ± 5.36^{b}	27.68 ± 3.06^{a}	33.13 ± 0.63^{ab}	32.97 ± 8.26^{ab}
Hetero:lymphocytes	0.17 ± 0.03^{c}	0.38 ± 0.04^{a}	0.30 ± 0.04^{b}	0.18 ± 0.20^{c}

Number of replicate per tanks per treatment=3.
Mean±standard deviation with different superscripts were significantly different (p<0.05).

The rates of antioxidant and immune parameters of *Oreochromis niloticus* infected with *Erysipelothrix pisciscicarius* and treated with Phytol are shown below in Table 4.

Significant differences were observed in all the parameters; SOD, Catalase, RBA, Lysosome activity (p<0.05). The highest antioxidant variable was observed in the fish treated with 25% Phytol while the lowest was recorded in the control treatment.

Table 4: Antioxidant and immune parameters of *Oreochromis nilotics* infected with *Erysipelothrix pisciscarius* and treated with Phytol.

Parameters	Phytol levels			
	Control (0.00mg/g)	1.75mg/g (25%)	3.50mg/g (50%)	7.00mg/g (100%)
Superoxide dismutase (1U/L)	14.76±2.21^{d}	37.43±0.74^{a}	28.97±2.75^{b}	18.53±0.69^{c}
Catalase (IU/L)	12.09±0.17^{b}	29.65±7.93^{a}	28.49±0.73^{a}	14.88±2.29^{b}
Lysozome activity (U mg/Protein)^{j}	3.56±0.63^{c}	10.84±1.02^{a}	8.07±0.15^{b}	2.99±0.28^{c}
RBA (mg/ml)	1.98±0.14^{c}	4.27±0.11^{a}	3.22±0.32^{b}	2.34±0.43^{c}

Number of replicate per tanks per treatment=3.
Mean±Standard deviation with different superscripts were significantly different (p<0.05).

Discussion

To understand the anti-bacterial activity of phytol against *Erysipelohris pisciscicarius* in *Oreochromis niloticus*, the fish were exposed to the bacterium in a laboratory controlled challenge trial. Immersion challenge was chosen to mimic the proposed natural infection route established, while fulfilling Koch's postulates. The result of this study showed the control group infected with *Erysipelothrix piscicarius* to have nectotizing dermatis in agreement with the work of Pormanski *et al*., 2018. About 70% mortality observed in this study is in agreement with the 83% mortality observed in Tiger barb (Chang *et al*., 2021).

The histopathological distortions observed in this study are similar to the observation of Chag *et al*. (2021) on gill. Influence of phytol as antibacterial properties was noticed in this study and is in concomitant with the works of Adeshina *et al*. (2018, 2019, and 2021), who should positive influence of phytobiotics against pathogenic bacteria. However, variation in the trend and dynamics of the performance may be attributed to the volatility of the compounds (phytol). Furthermore, increased in the survival rate of the fish gavaged with phytol as shown in the antioxidant and immune profiles is proportional the influence of the phytol on the fish in an inverse order.

In the current investigation, fish gavaged with phytol levels had considerably greater values of RBCs, Hb, Ht, WBCs, and platelets than the control group. This adds to the evidence that phytol enhanced the fish's hematological function. Fish with higher blood RBC, Hb, and Hct levels have better tissue oxygenation, which promotes better growth and health. According to the current study, the administration of phytols boosted the antioxidant capacity of Nile tilapia, which may have contributed to improvements in hematological parameters. Osmotic fragility test findings and decreased hemolysis and RBC destruction are both supported by antioxidants' ability to protect RBC membrane lipids from oxidative stress. Similarly, consuming diverse antioxidant chemicals boosted antioxidant defenses and reduced RBC hemolysis in many fish species (Hoseini *et al*., 2021). On the other hand, Nile tilapia gavaged with phytol zevels had

significantly lower levels of lymphocytes, heterocytes, monocytes, eosinophils, and basophils, whereas fish fed the control diet had the greatest amounts of these cells. Similar outcomes were shown in Nile tilapia fed green tea leaves, African catfish fed graded levels of Tridax procumbens and clove basil, and African catfish fed Tridax procumbens (Abdel-Tawwab *et al*., 2010; Abidemi-Iromini and Kolawole, 2019).

SOD and CAT are regarded as crucial enzymes involved in enzymatic processes that reduce fish oxidative stress. These enzymes may function to keep the body's redox equilibrium in check and reduce the imbalance of reactive oxygen species (Abdel-Daim *et al*., 2019). The fish treated with 25% phytol had the greatest levels of SOD and CAT at the conclusion of the experiment. Many secondary metabolites and bioactive phytol substances with antioxidant properties may be responsible for these effects (Beck *et al*., 2018). In various investigations on the use of herbal materials in fish meals, increases in the activity of these enzymes have been linked to the inhibition or alleviation of oxidative stress. For instance, dietary supplementation with Phoenix dactylifera fruit extract from the palm, *Silybum marianum* seed from the plant, *Artemisia annua* leaf extract from the plant, *Eichhornia crassipes* leaf extract from the plant, and flavonoids from *Allium mongolicum* from the plant have all significantly improved SOD and CAT activities as well as suppressed oxidative stress in numerous fish species (Hoseinifar *et al*., 2017).

Important innate immune indicators such as RB and LYZ activities have been utilized to assess fish innate immunity (Magnadottir, 2006; Secombes and Wang, 2012). Both elements contribute to the resistance against pathogen invasion. It has been found that phytol increased LYZ and RB activities, and that fish treated with 25% of phytol had the highest levels of these activities. According to earlier research, fish using plant-based food supplements (powder/extracts) exhibit higher LYZ and RB profiles (Adeshina *et al*., 2019; Adeshina *et al*., 2020; Abdel-Tawwab and El-Araby, 2021).

Conclusion

The study concluded that gavaging of 1.75 mg/g of phytol in protects Nile tilapia against *Erysipelothrix pisciscicarius* infection, enhances haematological and antioxidant profilesof the fish.

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