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Effects of *Moringa oleifera, Allium sativum, Zingiber officinale* on the haematological parameters and histopathological changes in visceral organs of *Clarias gariepinus* infected with *Pseudomonas aeruginosa*

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Abstract

Bacterial resistance to synthetic antibiotics is been a worrisome issue globally resulting in the search for alternative medicament for the treatment of bacterial infection in man, fish and other livestock. The study investigated the histological changes caused by selected medicinal plant extracts on sub-adults of the sharp tooth African catfish Clarias gariepinus experimentally infected with Pseudomonas aeruginosa. Phytochemical screening of test plants was done using standard methods. Agar well diffusion method was used to screen susceptibility of P. aeruginosa; ATCC 27853 to extracts of Moringa oleifera, Allium sativum, Zingiber officinale and oxytetracycline at 1000 mg/ml, 750 mg/ml and 500 mg/ml. Three fish samples from each group were tested for immune response and histopathological alterations. Saponins, terpenoids, flavonoids, cardiac glycoside, anthraquinone and alkaloids were present in test plants. Values of packed cell volume was 29.33%, 30.67%, 34.33%, 25.33% and 22.33% in fish exposed to oxytetracycline, Z. officinale, M. oleifera, A. sativum and untreated fish respectively. Similar trend was observed in the levels of red blood cell, haemoglobin and lymphocyte. White blood cell and neutrophil values were comparatively high in the untreated than treated fish. Organs of untreated fish showed degenerations in gill lamellae, gastric glands and hepatocytes while treated fish organs showed slight regeneration. The studied medicinal plant extracts showed significant antibacterial activity and compared with oxytetracycline in the treatment of P. aeruginosa diseases in fish. Use of these medicinal plants enhanced cultured fish immune responses.

Key words: *Allium sativum; Clarias gariepinus;* histopathology; immune response; *Pseudomonas aeruginosa*

Introduction

Poor quality of fish environment predisposes fish to several bacterial infections. Diseases and pathogens have increased due to intensification of the aquaculture industry, thereby constituting an integral part of the industry globally (Kolkovski and Kolkovski, 2011). The commonly cultured fish species in Nigeria aquaculture industry- *Clarias gariepinus*, though well adapted, its culture is often characterized by microbial infections including *Pseudomonas* species, an ubiquitous bacteria in this environment (Efuntoye *et al*., 2012). Characteristic clinical sign of *P. aeruginosa* infection in fish species is septicaemic hemorrhage around the mouth, opercula and ventral side of the body. With histology, lesions include shrinkage of hepatocytes, increase sinusoidal blood space in liver with vacuolization and necrotic changes in the kidney epithelial cell (Magdy *et al*., 2014). These changes provide clue to establish diagnosis. However, *P. aeruginosa* treatment poses other challenges including resistance to antibiotics.

The multidrug resistance of *P. aeruginosa* infections is increasing with worse clinical outcomes due to scarcity of efficacious

antimicrobial choices (Hirsch and Tam, 2010). The ability of this organism to resist antibiotics and the accompanying high mortality in infected fish species necessitated the initiation of new therapeutic measures in the fish farming industry to manage this pathogen. There is an upsurge of research on the use of vaccines, probiotics and immune stimulants from plant origin. Studies on new drug discoveries and efficacy of medicinal plants have been widely documented (Bello *et al*., 2012; 2014; Amrevuawho *et al*., 2016).

The medicinal plants used stem from previously documented studies on the medicinal properties of these plants, *Moringa oleifera* (Vergara-Jimenez *et al*., 2017; Othman and Ahmed, 2017), *Zingiber officinale* (Rong *et al*., 2009; Okiki *et al*., 2015) and garlic (Viswanathan *et al*., 2014; El-Gamal and El-Gazzar, 2017). Another reason for the choice of medicinal plants, is due to their availability in Nigeria. Despite the medicinal values and safety of these plants, there is paucity of information on their use in fish medicine. The study was therefore aimed at carrying out investigations to examine the immune stimulatory and antimicrobial activities of selected plants in *pseudomonas aeruginosa* sub-adult *C. gariepinus*.

Materials and methods

Fish sample collection and acclimatization

A total of one hundred and eighty sub-adult of the African catfish *C. gariepinus* weighing 250 ± 0.2 g, were purchased for the study and conveyed to the site of the experimental which was fish hatchery of the Federal University of Agriculture, Abeokuta, Nigeria. Before commencement of the experiment, the fish samples were acclimatized for 7 days during which they were fed (twice daily) with 3 mm Coppens fish feed (Alltech Coppens, Netherlands). In addition, microbial load in the intestine and muscle of fish sample was determined before commencement of study.

Collection and preparation of plant extracts

Seeds, bulbs and rhizome of *M. oleifera* (moringa), *A. sativum* (garlic) and *Z. officinale* (ginger) were used for this study. The plants parts were purchased from popular markets in Southwest, Nigeria. Aqueous preparation of extracts was according to Fatope *et al*. (1993). 100 g of plant parts were homogenized in water and allowed to stand for 72 hours with constant shaking. Filtration of homogenate was done using Whattman no. 1 filter paper. Concentration of filtrates was with rotary evaporator (5-50 L Rotovap, Re-La series, LABFREEZE, China) at temperature of 70 ^{o}C and was preserved at 4 ^{o}C in the refrigerator till further use.

Phytochemical analysis and Antimicrobial study of Plant Extracts

Sofowora (1993) and Trease and Evans (2002) methods for Qualitative phytochemical screening was adopted in this study.

Pseudomonas aeruginosa (ATCC 27853) was the bacteria used for antibacterial screening against the extracts. For the study, 0.5 MacFarland turbid overnight broth cultures of varying concentrations of the plant extracts at 1000, 750 and 500 mg/ml was used for testing. Agar well diffusion method was adopted for the study and interpretation of results was according to CLSI (2006) guidelines. The minimum inhibitory concentration (MIC) of extracts was evaluated using the broth dilution method described by Wiegand *et al.* (2008). Minimum bactericidal concentration (MBC) was determined from the MIC by sub culturing to agar plates that do not contain *P. aeruginosa*. The well with minimum volume of extract that showed growth was recorded as the MBC.

In both MIC and MBC determination, oxytetracycline was used as the control and was investigated concurrently with the

extracts.

Challenge Experiment

For the challenge experiment, an overnight incubated broth of 0.1 ml of 0.5 MacFarland of the test bacteria was administered orally into the experimental fish. Experimental fish were anaesthetized in 4 % chloroform before challenged with pathogen. Fish samples were placed in an empty bowl for 5 minutes and then released into the test environment (50 L rectangular aquarium filled to 40 L depth). Experimental fish were daily monitored for a 21 days period for signs of infection. Mortality was recorded (Thomas et al., 2014).

Experimental fish were randomly placed in 50 L aquarium at 15 fish/tank and replicated twice. Each fish was exposed by bath exposure to plant extracts and oxytetracycline of individual MICs at the observation of clinical signs of infection and negative control was exposed to ordinary water.

Haematological and histological analysis

For the analysis of the haemogram, 0.5ml of blood was drawn through the vertebral column of fish samples with two ml needle and syringe. Packed cell volume (PCV) was estimated with capillary heamatocrit analysis. Result gotten was reported in percentage of the total blood volume.

Haemoglobin concentration was determined by spectrophotometric method described by Franco, (1984) while red blood cell count was determined using Dacie and Lewis (1991).

The test adopted in this study for WBC count and differential was by counting the white cells using the haematocytometer. Blood samples were diluted in a 3 % aqueous solution of acetic acid in a ratio of 1:20 and gentian violet added. White cells were counted using x10 objective of the microscope in Neubauer counting chamber. White cells counted were expressed as cubic millimeter after multiplying by 50.

For white blood differentials, blood drop was spread thinly on a glass slide and left to air dry. It was then stained with Giemsa stain (May-Grunewald-Giemsa technique). 100 white cells were counted as percentage of each differential white cell using oil immersion objective microscopy. Identification of differential white cells was based on their nuclear stain, presence of granules and arrangement of the nucleus.

Histology of organs was by the paraffin method (Raji and Nourazi, 2010) while staining was as described by Bancroft and Cook (1994) Photomicrograph of desired parts of the tissues was taken using x400 magnification of the microscope.

Data Analysis

Mean ± Standard deviation (mean ± SD) was used to express data obtained from the study. Test for significance of the means was by the One-way analysis of variance (ANOVA). Post-hoc analyses to separate between treatment means were done using Duncan Multiple Range Test (Duncan, 1955).

Results and Discussion

Phytochemical screening of the plant extracts

Results obtained from the screening of plants for phyto compounds are as shown in Table 1. Compounds observed to be present in all of the plant extracts were Alkaloids, Terpenoids, and Cardiac glycosides.

Table 1: Qualitative phytochemical screening of plant extracts

Bio-active compounds	*M. oleifera*	*A. sativum*	*Z. officinale*
Tannins	_	_	_
Terpenoids	+	+	+
Flavonoids	-	-	+
Cardiac glycoside	+	+	+
Alkaloids	+	+	+
Saponin	_	+	+
Anthraquinone	-	+	-

Key: - indicate absence; + indicate presence

***In vitro* efficacy test**

P. aeruginosa susceptibility to all the test plant extracts showed the ability of the extracts to inhibit the growth of organism. Highest inhibition was recorded in *Z. officinale* extract. Minimum inhibitory concentrations (MIC) of 0.012, 0.013 and 0.011 g/l were recorded for *M. oleifera, A. sativum* and *Z. officnale* against the test bacteria pathogen. None of the extracts was observed to show bactericidal activity against the pathogen (Table 2).

Table 2: Antibacterial potential and minimum inhibitory concentrations of the extracts against the *Pseudomonas aeruginosa*

	Mean zone of inhibition (mm)			MIC (g/l)
Treatments	Concentrations (mg/ml)			
	1000	750	500	
M. oleifera	12.5±2.5^{a}	8.05±4.5^{b}	0.0	0.012
A. sativum	13.5±2.5^{a}	8.75±3.5^{b}	0.0	0.013
Z. officinale	16.0±5.0^{a}	10.0±3.5^{b}	0.0	0.011
Oxytetracycline	20.5±3.5^{a}	14.5±4.5^{b}	9.0±4.0^{c}	0.007

Values are expressed in means ± SE. Means having same letter (a) in the same column are not significantly different at p>0.05.

The result of antibacterial susceptibility of the extracts revealed all the extracts prevented the growth and survival of *P. aeruginosa* with none showing bactericidal activity. The present finding agreed with the work of Ankri and Mirelman (1999); Amagase (2006) and Mohammed (2013). They posited that a dose dependent antibacterial activity was observed when *A. sativum* was screened against *P. aeruginosa* and some bacteria organism. Similarly, ethanol extract of *Z. officinale* was indicated to prevent the growth and survival of some gram-negative and -positive bacteria organisms (Tan and Vanitha, 2004). The study of Habsah *et al*. (2000) however indicated the ability of ginger to kill *P. aeruginosa* (bactericidal), which was not the case with the finding of this study. The report of the study of Vander and Vlietnck (1991) and Suarez *et al*. (2005) was corroborated by this study as they reported that *M. oleifera* showed strong antibacterial activity against *P. aeruginosa* and some other bacteria organism. This activity could be linked to the presences of the phyto-compounds present in the different plants used for this study.

Haematological parameters of infected *C. gariepinus* sub-adult after exposure

Remarkable increase was observed in some of the red blood cell parameters of the test fish in the treatment groups than the

untreated fish (control group). Levels of WBC and neutrophil were observed to reduce in treatment groups Table 3. The RBC parameters of the experimental treated groups were increased than in the untreated samples. These findings were similar to what was indicated by Sivagurunathan *et al*. (2011) when they estimated the RBC parameters of fish infected with *P. aeruginisa* and fed experimental diets containing ginger and turmeric. Fazlolahzadeh *et al*. (2011) also gave an indication that RBC parameters of *Oncorhynchus mykiss* (rainbow trout) was increased after feeding with experimental diets containing varying inclusion rate of *A. sativum*. Similarly, Sahan *et al*. (2016) reported increased in these blood parameters in *Aeromonas hydrophila* infected *Oreochromis niloticus* fed varying levels of ginger diet. Hammed *et al*. (2015) gave similar report on the effect of *M. oleifera* in *C. gariepinus* infected with *A. hydrophila*.

Table 3: Mean values of blood parameters of *Pseudomonas aeruginosa* infected *Clarias gariepinus* sub-adult exposed to oxytetracycline and plant extracts

Blood Parameters	*M. oleifera*	*A. sativum*	*Z. officinale*	Oxytetracycline	Infected
PCV (%)	34.33±2.1^{b}	25.33±0.9^{ab}	30.67±3.7^{ab}	29.33±4.2^{ab}	22.33±0.3^{a}
HB (g/dl)	9.67±0.5^{c}	8.07±0.2^{abc}	9.20±0.8^{bc}	9.57±1.2^{bc}	6.97±0.2^{a}
RBC (x10^{12}/L)	1.90±0.3^{ab}	1.60±0,1^{ab}	1.90±0.3^{ab}	1.93±0.2^{b}	1.27±0.1^{a}
WBC (x10^{9}/L)	14.03±1.8^{abc}	11.97±0.9^{ab}	14.63±1.9^{abc}	14.93±1.7^{bc}	17.05±0.5^{c}
NEU (%)	33.67±7.6^{a}	37.33±3.5^{ab}	34.33±4.4^{ab}	35.00±4.0^{ab}	47.33±1.2^{b}
LYM (%)	66.67±8.4^{b}	61.33±2.7^{ab}	65.33±4.7^{ab}	64.00±4.0^{ab}	52.33±0.9^{a}

Values are expressed in means ± SE. Means having same letter in the same row are not significantly different at p>0.05.

Key: PCV = Packed Cell Volume, HB = Haemoglobin, RBC= Red Blood Cell, WBC = White Blood Cell, NEU= Neutrophil, LYM = Lymphocyte

Increase in these RBC parameters after instituting treatments suggests that these plants contain erythropoietin-like agent(s) (Ofem *et al*., 2012) which confers on them the ability to produce RBC (erythrocytes). This could be attributed to the phytochemicals in the plants (Chiang *et al*., 2003; Lopez and Urias-Silvas 2007; Punturee *et al*., 2005). Adeoti *et al*. (2009) however reported a decrease in the RBC parameter (PCV) of *Klebsiella pneumonia* infected rats fed with experimental diets containing garlic and ginger singly. This variation could be due to the physiological differences between the two species of the experimental animals used in the studies (Toutain *et al*., 2010).

Lowering of the mean value of the WBC count and white cell differential count of experimental treated fish could suggest that all the medicinal plants used in this study were able to combat the invading pathogen. Amrevuawho *et al*. (2016) described similar response of the total and differential WBC. However, the findings of this study did not agree with what was reported by Sivagurunathan *et al*. (2011) for *P. aeruginosa* infected *Cirrhinus mrigala* and fed experimental diets formulated with concentrations of *Z. officinale* and turmeric. The reason for this disagreement could be attributed to the duration of exposure and environmental condition in which the experimental fish were kept.

Histopathological changes in fish organs

After 21 days of exposure, challenged experimental fish showed various clinical signs to include: tail and fin rot, superficial lesions in

skin and skin discoloration and ulceration. Varying degree of recovery was observed in the organs in the experimental treated groups. *M. oleifera* and ginger treatment showed normal appearance of the gills with no visible injury. In the garlic group, the gill lamellae were observed to be moderately thickened (Figure 1). Liver of fish samples in the treated groups showed varying types cytoplasmic vacuolation of the hepatocytes (Figure 2). Stomach of the experimental fish in the different treatments revealed varying degree of recovery compared to the control (Figure 3).

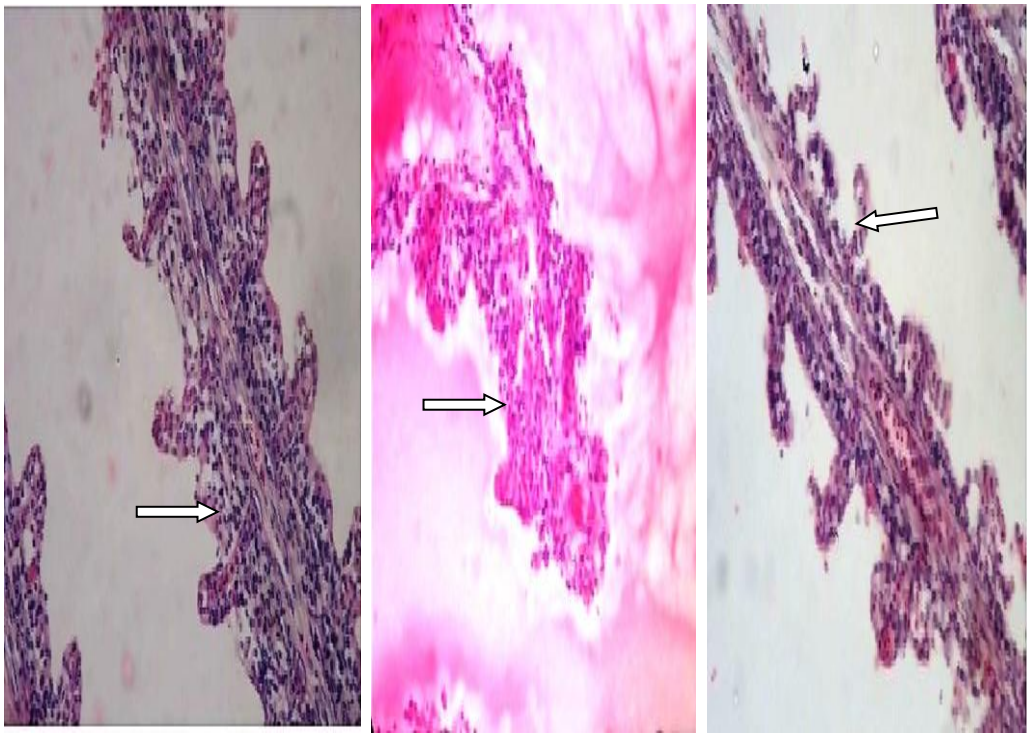


Figure 1: Section of the gill of treated and untreaed infected *C. gariepinus*

a. *A. sativum* treated fish showing thickening due to degeneration and injury in the epithelial cells of gill lamellae with infiltration of inflammatory cells (arrow) b. untreated fish showing marked loss and sloughing off of the primary and secondary gill lamellar c. oxytetracycline treated fish showing moderate loss of the gill lamellae (arrow) (x400; H & E)

Changes observed in the gills, liver and stomach of sample diseased fish from the histopathology is a clear indication that upon invasion, *P. aeruginosa* attacks the internal organs of fish. The various pathological changes ranging from loss of gill lamella, damage to the hepatocytes,

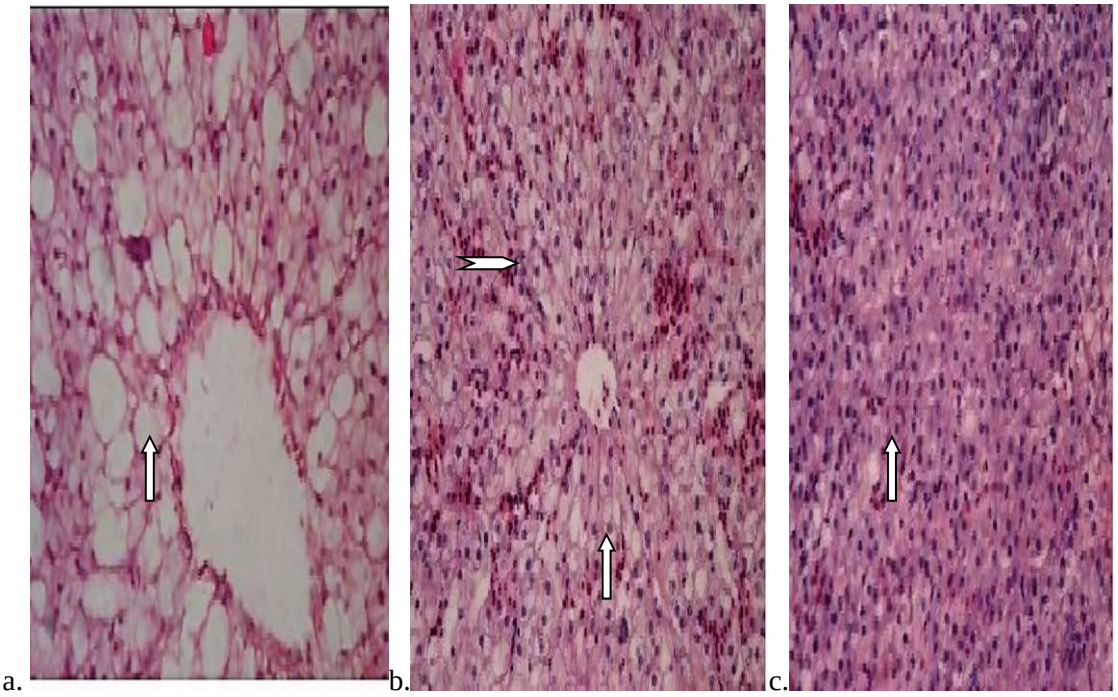
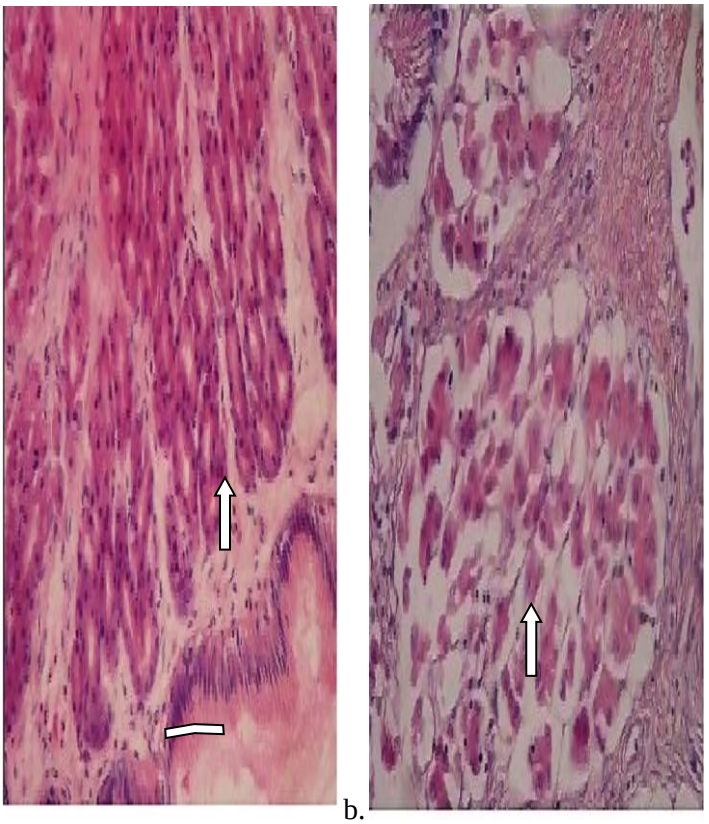


Figure 2: Section of the liver of *C. gariepinus* infected with *P. aeruginosa*
a. *M. oleifera* treated fih showing multiple foci of hepatocytes that contain large cytoplasmic vacuoles (arrow) b. untreated fish showing severe vacuolar degeneration of hepatocytes (arrow) and congestion of the sinusoid (arrow head) c. oxytetracycline treated showing regeneration of the hepatocytes (arrow) (x400; H & E)

congestion of the sinusoid to shortened rugae and damage to the gastric gland in the stomach can be accredited to the ability of the organism to produce harmful substances such as pigments, hydrocyanic acid, phytotoxic factor, proteolytic enzymes, and others which activities can result to necrosis of liver, haemorrhage as reported by other workers (Magdy *et al.*, 2014).



a. b.

Figure 3: Section of the stomach of treated and untreated infected *C. gariepinus*
a. *Z. officinale* treated fish showing regeneration of cryptal glands (arrow) and the mucosal epithelium (arrow head) b. untreated fish showing severe necrosis of glandular epithelial cells (arrow) (x400; H & E)

The regeneration observed in the gill lamellae of the treated fish groups suggests that the organ can recover from the pathological lesions produced by *P. aeruginosa* when treated. Similar observation has been reported by Amrevuawho *et al*. (2016). Chronic bronchitis with abundant eosinophilic infiltration was observed in gills of rainbow trout infected and treated with *β*-lactams has however been reported earlier (Saaveedra *et al*., 2004). Differences could be due to mode of action of the antibiotics (Dowling *et al*., 2013).

Conclusion and Recommendations

The medicinal plant extracts showed significant antibacterial activity and compared with oxytetracycline in the treatment of *P. aeruginosa* diseases in fish. Thus, they can be used as substitute to synthetic antibiotics (oxytetracycline) in the treatment of *P. aeruginosa* diseases in fish. Use of these medicinal plants enhanced cultured fish immune responses.

In recommendation, there is need for farmers to be educated on the medicinal properties of these available medicinal plants and on how they can be included in fish diets so as to boost the immune response of cultured fish to enable them fight against bacterial infection.

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