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FFECT OF HERBAL BASED IMMUNOSTIMULANT DIETS FOR DISEASE CONTROL IN AFRICAN CATFISH *Clarias gariepinus* AGAINST *Aeromonas hydrophila* INFECTIONS.

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Abstract
The effect of herbal immunostimulants enriched diets was studied on some immune response parameters and disease resistance of African catfish *Clarias gariepinus* in south south Nigeria. One hundred and sixty *160* fish, average weight 100 ± 1. 4 g^{-1} were randomly divided into three *3* groups. One group was immunized with formalin inactivated vaccine with a booster dose after 14 days (2 weeks) post immunization. The second group was subdivided into two (2) treatment groups and fed with *Allium sativum* and *Ocimum sanctum* supplemented diets respectively for two weeks (14 days). The third group was fed with commercial diet supplemented with sterile phosphate buffer saline *PBS* and considered as control. Results showed that the oral administration of *Allium sativum* and *Ocimum sanctum* enriched diets and the immunized group were able to significantly enhanced lysozyme activity, serum bactericidal activity, as compare to the control. The study thus showed that oral *Allium sativum* and *Ocimum sanctum* supplemented diets could be potential candidates for herbal immunostimulants for African catfish rearing.
Keywords: *Allium sativum*, *Ocimum sanctum*, immunostimulants and *Clarias gariepinus*

1. Introduction

The African catfish, *Clarias gariepinus* is of the Claridae family and widely spread in the waters of tropical Africa, India, and Asia. They are the most cultured fish in Africa and Nigeria in particular, because of their ability to survive wide variety of environmental conditions and reproduction in captivity. More so, their physiological attributes such as fast growth, high fecundity, efficient breathing organs, disease resistant and survival in captivity endear it to many culturists and makes it a commercial important fish in the country (Kestemont et al., 2007 and Safina *et al*, 2013).

Catfish *Clarias sp* like every other fish are sensitive to infectious agents through attendant nonspecific and specific immune response or reactions. They overtly relied on nonspecific immune mechanism for survival (Sakai, 1999). Immunostimulants are compounds with capacity to heighten the immune resistance to disease by enhancing the nonspecific and specific or adaptive defense mechanisms (chakrabarti and Rao, 2006). The use of herbal based immunostimulants in aquaculture spells a promising alternative to the use of antibiotics and vaccine. Most immunostimulants which have been screened for application in aquaculture include among others, glucan (Chen & Ainsworth, 1992), tunicate extract (McCumber et al., 1991), allium sativum (Nya & Austin, 2009), Ginger (Nya & Austin, 2009), Levamisole (Findlay & Munday, 2000; Siwicki et al., 1989), lactoferrin (Sakai, 1999), Vitamin C (Sahoo & Mukherjee, 2003), Oligonucleotides from yeast RNA (sakai et al., 2001), lipopolysacharides *LPS* (Nya & Austin, 2010). These and other plants derivatives are shown to initiates or activate innate defense mechanisms that may results in production of various components of immune properties such as phagocytic activity, lysozymes and complement activities, production of antimicrobial molecules and macrophages.

Herbal based immunostimulants are environmental friendly, biodegradable, cost effective and bioavailable when used (Nya & Austin, 2009). Garlic, *Allium sativum* in particular, has a history of dietary and medicinal applications as an anti-infective agent (Reuter, Koch & Lawson 1996; Lawson 1998). Indeed, many experimental and clinical effects of fresh and oven-dried garlic preparations have been documented, and include use of garlicin and crushed garlic (Corzo-Martinez, Corzo & Villamiel 2007; USDA 2007). Evidence of its value includes inhibition of bacterial growth (e.g. Pszczola 2002) and fungi growth (Amer, Taha & Tosson 1980; Gupta & Porter 2001). In use, garlic leads to stimulation of immune functions in humans and in fish (Sahu, Das, Mishra, Pradhan & Sarangi, 2007). In the other hand, Sweet basil *Ocimum sanctum*, possesses a variety of biological activities. Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), the active compound present in Ocimum sanctum L., has been found to be largely responsible for the therapeutic potentials of this agent (Prakash and Gupta, 2005). Extracts of various parts of *Ocimum* plant on immune system, reproductive system, central nervous system, cardiovascular system, gastric system, urinary system and blood biochemistry have been described as responsible for the therapeutic significance of this plant in management of various ailments

(Prakash and Gupta, 2005).
Consequently, the aim of this study was to examine the protective effects of dietary *Allium sativum* and *Ocimum* in African Catfish *Clarias gariepinus* against pathogenic infections

2. Materials and Methods

2.1. Feeds preparation and Feeding regimes

Oven-dried garlic bulbs and fresh *Ocimum* leaves were obtained from a local market in Uyo, Nigeria. The leaves and the bulb was crushed into small grains using a household garlic press, and mixed directly with commercial fish feed (Akwafeed; livestock feed company Nig. Ltd) to achieve 0 g^{-1} (control), 1.0 g^{-1} per 100 g^{-1} of feed. The modified feed was stored in screw cap bottles at room temperature until use. The experimental fish were fed twice daily to satiation for 14 days.

3. Experimental design

Three hundred *300* fish average weight 100 ± 1. 4 g^{-1} obtained from private fish farm in *South South Nigeria*. Fish were acclimatized in the aquarium for one week prior to the experiment. The water quality parameters were temperature *25 ±1 °C*, dissolved oxygen *8–9 mg/L*., pH *7.4 ±0.2* , NH_3 *<0.02 mg/L* and NO_2 *< 0.1mg.L*. Change of water was done at the rate of 50% daily. One hundred and Sixty *160* fish were used in the experiment. These fish were randomly divided into three *4* groups of *40* fish each and placed in a *500* litres rubber tanks. One group was immunized with formalin inactivated vaccine with a booster dose after 14 days (2 weeks) post immunization. The second and third treatment groups were fed with *Allium sativum* and *Ocimum sanctum* supplemented diets respectively for two weeks (14 days). The fourth group was fed with commercial diet supplemented with sterile phosphate buffer saline *PBS* and considered as control.

3.1. Vaccine preparation

Aeromonas hydrophila isolate was sourced from Biotechnology and Genetics laboratory, Akwa Ibom State University, Nigeria. The isolate was grown in tryptone soy broth at 30°C for 24 h and centrifuged at 800 g for 15 min. It was washed and resuspended in sterile *PBS* and were then inactivated using 1% formalin for 1 h before being washed *3* times in *PBS* and stored for use.

3.2. Challenge Experiment

Bacterial broth cultures were prepared in tryptone soy broth (TSB; Oxoid) with overnight incubation at 30°C. Then, the cultures were centrifuged at 3000 g for 10 min at 4°C, before the cells were washed twice in phosphate-buffered saline (PBS; Oxoid), pH 7.4, and the pellets resuspended in fresh buffer. The concentration was adjusted to 10^7 cells ml as determined by means of a haemocytometer slide (Improved Neubauer Type; Merck) at a magnification of 40 on a light microscope. Fish were challenged by intraperitoneal injection (i.p) with 0.1ml^{-1} suspensions of the pathogen (suspensions of A. hydrophila), 24 h after stopping feeding, and mortalities were recorded over 14 days, and any dead or moribund fish were examined bacteriologically to confirm the presence of A. hydrophila (after Austin & Austin 1989), The relative percentage survival (RPS) was calculated according to Amend (1981).

3.3. Growth calculation

Growth and nutrient utilization were determined according to Bag et al., (2011) in terms of feed intake (FI), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), energy retention (ER) and hepatosomatic index (HSI) as follows, briefly:
FI (g $fish^{-1}$ day^{-1}) = Total feed intake per fish/number of days.
SGR (% day-1) = 100 × (ln [final body weight]- ln[initial body weight])/no. of Days
FCR = feed intake/live weight gain
PER = live weight gain/crude protein intake.
ER (%) = 100 × (final fish body energy - initial fish body energy)/gross energy intake
HSI (%) = 100 × (liver weight/total body weight)

3.4. Serum collection

Blood samples were collected at the end of the feeding and immunization regimen. Ten *10* fish were sampled randomly from each experimental group, anaesthetized with MS 222 (tricaine methanesulfonate,TMS) at 0.1 mg L^{-1} before blood was collected by venepuncture, and transferred into vacuette tubes containing heparin as anticoagulant (Greiner) to prevent clotting. Sera samples were separated by centrifugation at *2000 g for 10* min and stored in separate Eppendurf tube (Greiner) at - 20 °C until used.

3.5. Relative Percentage survival (RPS)

Virulent strain of *A. hydrophila* was used for disease resistance assay. Ten *10* fish from each treatment group were intraperitoneally challenged with the bacterial suspension (2.1 × 10^7cfu per fish) and mortality of challenged fish was recorded daily for 14 days. The cause of death was ascertained by re-isolating the infecting organism from kidney and liver of dead fish after Austin & Austin (1989).

3.6. Serum bactericidal activity

Bactericidal activity was studied following procedure by Kajita et al. (1990) with slight modification. Sera samples were diluted three times with phosphate-buffered saline (PBS; Oxoid), pH 7.4, (containing 0.5 mM ml-1 Mg2+ and 0.15 mM ml-1 Ca2+). *A. hydrophila* (live, washed cells) suspended in the same buffer at concentration of 10^5 colony forming unit *CFU* ml. The diluted sera and bacteria were mixed at 1:1 (v/v), incubated for 90 min at 25 °C with occasional shaking. Control group containing bacterial suspension was also subjected to the same treatment. The numbers of viable bacteria was then calculated by counting the colonies from the resultant incubated mixture on TSA plates after 24 h incubation in duplicate. The bactericidal activity of test serum was expressed as percentage of *CFU* in test group to that of control group.

3.7. Lysozyme activity assay

Serum lysozyme activity was measured in accordance to Ellis (1990) with a slight modification. Briefly, 10 μl^{-1} of individual serum was mixed with 200 μl^{-1} of a *Micrococcus lisodeichticus* (Sigma) suspension at 0.2 mg ml^{-1} in phosphate buffer saline *PBS* (pH 7.4). The mixture was incubated at 27 °C, and its optical density was measured after 1 and 6 min at 530 nm using an ELISA plate reader. One unit of lysozyme activity was defined as the amount of enzyme causing a decrease in absorbance of 0.001 min^{-1}ml serum.

3.8. Alternative complement activity

Alternative complement activity was measured following the procedure of Yano (1992), using rabbit red blood cells (RaRBC). Briefly, RaRBC were washed and adjusted to $2x10^8$ cell ml^{-1} in ethylene glycol tetra acetic acid-magnesium-gelatin veronal buffer (0.01 M). A 100 μl^{-1} of RaRBC suspension was lysed with 3.4 ml distilled water and the absorbance of the haemolysate was measured at 414 nm against distilled water to obtain the 100% lysis value. The test serum was appropriately diluted with 3.4 ml distilled water and allowed to react with 0.1 ml^{-1} of RaRBC in test tubes. After incubation at 20 °C for 90 min with occasional shaking, 3.15 ml^{-1} of a saline solution was added to each tube and were centrifuged at 1600 *g* for 10 min at 4 °C. The optical density of supernatant was measured at 414 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of serum added.

The volume yielding 50% haemolysis (ACH50) was determined and used for calculating the complement activity of the sample as follows: ACH50 value (units ml^{-1}) =1/K x (reciprocal of the serum dilution) × 0.5 where K is the amount of serum (ml^{-1}) giving 50% lysis and 0.5 is the correction factor since this assay was performed on half scale of the original method.

4. Statistical analysis

Values for each parameter measured were expressed as arithmetic mean ± standard error (SE). The effect of herbal based immunostimulants (*Allium sativum* and *Ocimum sanctum)* supplemented diets on some immune response parameters and disease resistance of African catfish *Clarias gariepinus* were tested using one-way ANOVA, and a comparison of the mean values was made by using Duncan's multiple range tests (Duncan 1955), at 5% level of significance. The software programme SPSS (version. 14.0) for Window was used for the analysis.

5. Results and Discussion

The protective effect of *Allium sativum* and *Ocimum sanctum* enriched diets on African catfish *Clarias gariepinus* challenged with virulent *Aeromonas hydrophila* inoculum demonstrated that the oral administration of these plant derivatives are able to stimulate some non-specific immune mechanisms of African Catfish *Clarias gariepinus*, thus are better described as herbal based immununostimulants.

Relative percentage survival (RPS)*:* The mortality rate in *Allium sativum* and *Ocimum sanctum* treated groups during 14 days post-challenge period is shown in Fig.1. Levels of mortality were 40%, in immunized groups; 10% and 5% in fish fed with feed supplemented with *Ocimum sanctum* and *Allium sativum* diets respectively. Significant changes was also seen in treated fish as compared to the control ($P>0.05$). Also, mortality in non-immunized (control) groups were 86.7%.. The relative percentage survival (RPS) was significantly high in *Allium sativum* and *Ocimum sanctum* supplemented diet ($P<0.05$). Resistance to *A. hydrophila* infection paralleled significantly in the immunized and in both *Allium sativum* and *Ocimum sanctum* treatment groups. However, the non immunized (control) group did not showed reduced mortality after experimental challenged with pathogen. In a similar works, resistance against bacterial infection in fish was observed after oral administration of some herbal based therapeutics such as *Aloe vera* (Kim et al. 1999; Alishahi et al. 2010), Eclipta alba (Christybapita et al. 2007) and *Solanum trilobatum* (Divyagnaneswari & Cristybapita 2007).

Growth parameters of African catfish: The highest weight gain was observed in the treatment group administered with *Allium sativum* supplemented diet. The results showed high acceptability for the *Allium sativum* by cultured Africa catfish. This was possibly due to the high palatability of feed and preference of the fish to consume it as their potential food. The low FCR of *Allium sativum* indicates the bioavailability nature of

supplemented feed and that fish can easily digest the feed and convert the nutrients component to their body tissue. The tested value of FCR showing lower ratio indicates a favorable effect for market economy, due to the quality of the product. The protein efficiency ratio was significantly ($P<0.05$) high in *Allium sativum* feed treatments than *Ocimum sanctum* and immunized groups treatments which indicates the good quality of their protein as well. The highest ER value gain indicates the good efficiency of *Allium sativum* in terms of energy retention capacity. Similar reports were obtained by Nya & Austin, (2009) and Bag et al., (2011) among others.

Serum lysozyme activity: The serum lysozyme activity was increased significantly in the fish fed for 14 days with feed supplemented with *Allium sativum* and *Ocimum sanctum* and in immunized fish only (Fig 2) but not in non-immunized groups ($P < 0.05$). The highest lysozyme activity was observed in the immunized fish and follow by group fed with *Allium sativum* supplemented diet for 14 days. This work showed the result of serum lysozyme activity was significantly increased in fish fed with *Allium sativum* and *Ocimum sanctum* supplemented feed, and confirmed same in immunized fish as compared to non-immunized (control) groups. Lysozyme is said to be an enzyme that splits peptidoglycan in the bacterial cell wall and thus non-specifically inhibits the growth of infectious microorganisms. Therefore an elevated serum lysozyme activity specifies improved immune mechanism. This increase serum lysozyme activity obtained is in line with elevated lysozyme level obtained in large yellow croaker (Jian & Wu 2003) and common carp (Jian & Wu 2004) after feeding the fish with various herbal extracts**.** Moreso, several studies have demonstrated immunostimulants, probiotics and vaccines to potentially enhance the serum lysozyme activity in fish (Paulsen et al. 2001 and Villamil et al. 2003).

Alternative complement activity: Fish fed with *Allium sativum, Ocimum sanctum* supplemented feed, and immunized fish groups did showed changes in their alternative complement pathway compared to the non-immunized control groups ($P<0.05$). It is arguable that the mode of action of *Allium sativum* and *Ocimum sanctum* on the immune system of African Catfish could involve stimulation of the complement cascade (Janeway 1993). In their studies Nya & Austin (2009), reported Lectin as the most abundant protein in garlic (Fenwich & Hanley 1985), and is considered to bind to bacterial cells to trigger the complement cascade and subsequently phagocytosis (Magnadottir 2006), thus modulating the immune mechanism of the host. However, mannose-binding lectin (MBL) is regarded as the most abundant plant protein and has been implicated in immune modulations of aquatic organisms. Many workers have reported on the effect of plant based immunostimulators on the complement activity (Nya & Austin, 2009). Some reports confirmed increased complement activity as shown by Bagni et al. (2005) and Cheng et al. (2007), Conversely, others reports showed lack of significant effect on complement activity after administration of immunostimulants (Robertsen 1999). In this study *Allium sativum* and *Ocimum sanctum* did induced changes in alternative pathways of complement activity compare to control group.

In conclusion oral administration of *Allium sativum* and *Ocimum sanctum* enriched diets were able to enhance some immunological parameters of African catfish including lysozyme activity, complement activity, serum bactericidal activity up to 14 days post-challenged. Also, the relative percentage survival (RPS) of fish fed these herbal based immunostimulants was increased after challenged with virulent strain of *A. hydrophila* .Therefore it may be useful to use *Allium sativum* and *Ocimum sanctum* supplemented at 100 mg kg^{-1} diet for this fish species, particularly during their stress conditions. However the actual immunostimulatory active compounds in these herbs calls for further investigations.

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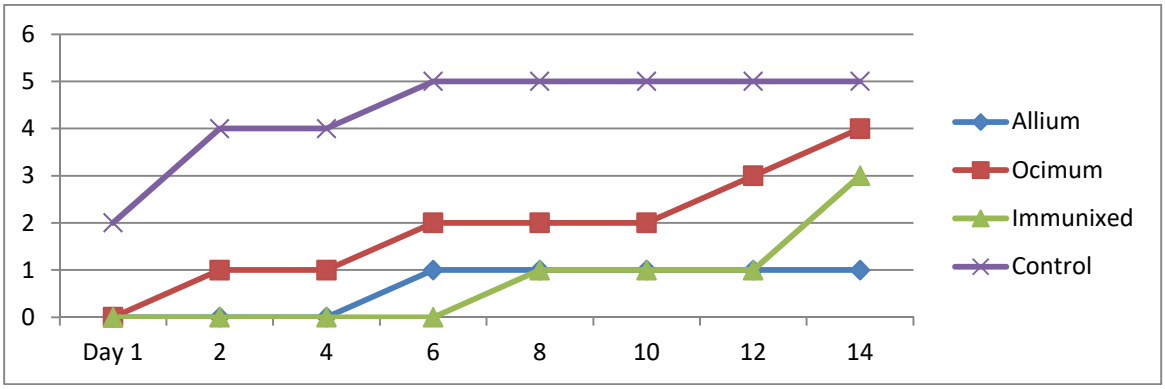
Relative percentage survival (RPS)

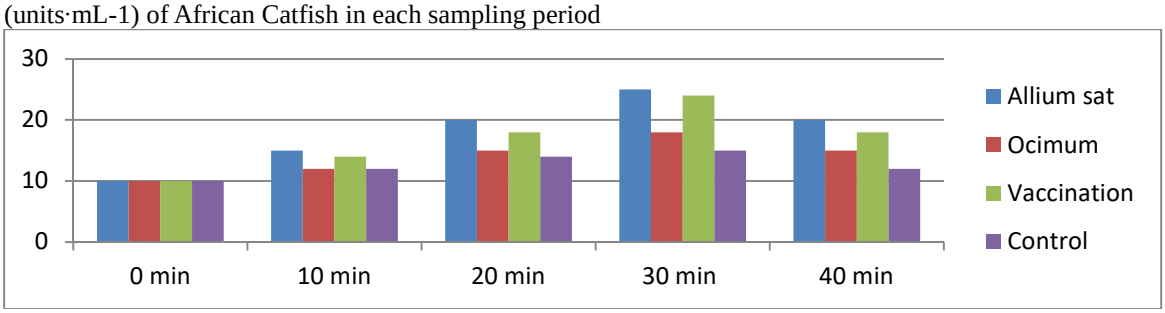
Fig. 1. Cumulative mortality of African Catfish post bacterial challenge after oral administration of supplemented feeds, Immunized fish and non-immunized dish (Control).)..



The results represent Mean ± SD (n = 20) in each treatment. Statistical significance between treatments: (P < 0.05.

Lysozyme activity:

Fig.2.The effects of *Allium sativum* and *Ocimum sanctum and immunization* on serum lysozyme activity (units·mL-1) of African Catfish in each sampling period



(Values are Mean ± SD, n= 10).

Table 1. Growth parameters of African catfish fed with *Allium sativum*, *Ocimum sanctum* supplemented diets , immunized fish and non-immunized fish (Control).

Treatment	Wt. gain g	FCR	SGR	PER	HIS %
Allium sativum	1.26.g	1.5±0.4	1.6±0.4	1.2±0.2	2.73
Ocimum sanctum	1.15	1.3±0.5	1.9±0.3	1.5±0.4	2.5
immunized	1.13	1..0±0.7	1.4±0.1	1.7±0.5	2.3
Control	110	2.3±0.9	1.2±0.3	0.9±0.07	2.0

Data expressed as mean _ SE, P < 0.05, n = 10. SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio and HSI, hepatosomatic index

Alternative complement activity

Table 2. The effect of *Allium sativum* and *Ocimum sanctum and immunization* on alternative complements activity of African Cat fish

Treatment	5µL^{-1}	10µL^{-1}	15µL^{-1}
Allium sativum	22.01	22.1	22.5
Ocimum sanctum	22.1	22.3	22.7
Immunization	22.3	22.4	22.5
Control	22.0	22.1	22.1

(Mean±SD, n=10). Means in the same column were not significantly different (P<0.05) from control.

References

Amend D.F. (1981). Potency testing of fish vaccines. Developments in Biological Standardization 49, 447–454,

Amer M., Taha M. & Tosson Z. (1980) Effect of aqueous garlic extract on the growth of dermatophytes. International Journal of Dermatology 197, 285–287.

Alishahi, M., Ranjbar, M. M, Ghorbanpour, M, Peyghan, R, , Mesbah, M and Razi jalali, M.(2010). Effects of dietary *Aloe vera* on specific and nonspecific immunity in the *Cyprinus carpio*, *International Journal of Veterinary Researches* 2:85-94.

Austin B. & Austin D.A. (1989). Microbiological Examination of Fish and Shellfish. Ellis Horwood, Chichester.

Bag, M.P., Mahapatra, S.C., Rao P.S. and Chakrabarty D. (2011). Making aquatic weed as potential feed for Nile tilapia (*Oreochromis niloticus* L.) and its impact on fatty acid profile. International Research of Pharmacy and Pharmacology, 1(8):194-202.

Bagni, M., N. Romano, M.G. Finoia, L. Abelli, G. Scapigliati, P.G. Tiscar, M. Sarti and G. Marino. (2005). Short- and long-term effects of a dietary yeast β-glucan (Macrogard) and alginic acid (Ergosan) preparation on immune response in sea bass (*Dicentrarchus labrax*). *Fish & Shellfish Immunol*ogy 18:311–325.

Chakrabarti, R. and Rao, Y.V. (2006). Achyranthes aspera stimulates the immunity and enhances the antigen clearance in *Catla catla*. *International Immunopharmacology* 6:782-790.

Chen, D. and Ainsworth, A.J. (1992). Glucan administration potentiates immune defense mechanisms of channel catfish (*Ictalurus punctatus* Rafinesque). *Journal of Fish Disease*s 15:295-304.

Cheng, A.C., Tu, C.W.,. Chen, Y.Y, Nan, F.H. and. Chen, J.C. (2007). The immunostimulatory effects of sodium alginate and κ-carrageenan on orange-spotted grouper *Epinephelus coicoides* and its resistance against *Vibrio alginolyticus*. *Fish & Shellfish Immunology* 22:197–205.

Christybapita, D., Divyagnaneswari, M and. Dinakaran, M.R. (2007). Oral administration of *Eclipta alba* leaf aqueous extract enhances the non-specific immune responses and disease resistance of *Oreochromis mossambicus*. *Fish & Shellfish Immunology* 23:840-852.

Corzo-Martinez M., Corzo N. & Villamiel M. (2007). Biological properties of onions and garlic. Trends in Food Science & Technology 18, 609–625.

Divyagnaneswari, M. and D.R. Christybapita (2007). Enhancement of nonspecific immunity and disease resistance *in Oreochromis mossambicus* by *Solanum trilobatum* leaf fractions. *Fish & Shellfish Immunology* 23:249-259.

Duncan, D.B. (1955). Multiple range and multiple 'F' tests. Biometrics 11, 1–42.

Ellis, A.E., Stolen, J. S, Fletcher, D. P, Anderson, B.S. and. Robertson, B.S (eds.) (1990). Techniques in Fish Immunology. SOS Publication, Fair Haven, New Jersey, 101–103.

Fenwich G.R. & Hanley A.B. (1985). The genus Allium. CRC Critical Reviews in Food Science and Nutrition 22, 199–377.

Findlay, V.L. and Munday, B. L (2000). The immunomodulatory effects of levamisole on the nonspecific immune system of Atlantic salmon (*Salmo salar* L.). *Journal of Fish Disease*s 23:369-78.

Gupta N. and Porter T.D. (2001). Garlic and garlic derived compounds inhibit human squalene mono-oxygenase. Journal of Nutrition 131, 1662–1667.

Janeway, C.A.(1993). How the immune system recognizes invaders. Scientific American 269, 73–79.

Jian, J. and. Wu, Z. (2003). Effects of traditional Chinese medicine on nonspecific immunity and disease resistance of large yellow croaker, *Pseudosciaena crocea* (Richardson). *Aquaculture* 218:1–9.

Jian, J. and Wu, Z. (2004). Influences of traditional Chinese medicine on nonspecific immunity of Jian carp (*Cyprinus carpio* var. Jian). *Fish & Shellfish Immunology* 16:185–191.

Kestemont, P; Toko, I; Fiogbe, E.D and Koukpode, B (2007). "Rearing of African catfish (*Clarias gariepinus*) and vundu catfish (*Heterobranchus longifilis*) in traditional fish ponds (whedos): effect of stocking density on growth, production and body composition," *Aquaculture*, vol. 262, no. 1, pp. 65–72, 2007.

Kim, H.K., W.J. Hwang and S.C. Bai. (1999). Resistance to *Vibrio alginolyticus* in juvenile rockfish *Sebastes schlegeli* fed diets containing different doses of *aloe*. *Aquaculture* 180:13–21.

Lawson L.D. (1998). Garlic: a review of its medicinal effects and indicated active compounds. In: Phytomedicines of Europe, their Chemistry and Biological Activity, ACS Symposium Series 691, (ed. by L.D. Lawson & R. Bauer), pp. 176–209. American Chemical Society, Washington, DC.

McCumber, L.J., R. Trauger and M.M. Sigel. 1991.. Modification of the immune system of the American eel (*Anguilla rostrata*). by ETE. Dev Biol Stand 49:289-294.

Magnadottir B. (2006). Innate immunity of fish (overview). Fish and Shellfish Immunology 20, 137–151.

Nya E.J. & Austin B. (2009). Use of garlic, Allium sativum, to control Aeromonas hydrophila infections in rainbow trout, Oncorhynchus mykiss (Walbaum). Journal of Fish Diseases 32, 963–970.

Nya, E J and Austin,B (2010). Use of bacterial lipopolysaccharide (LPS) as an immunostimulant for the control

of Aeromonas hydrophila infections in rainbow trout Oncorhynchus mykiss (Walbaum). Journal of Applied Microbiology 108 (2010) 686–694

Paulsen, S.M., Engstad, R. E and Robertsen, B. (2001). Enhanced lysozyme production in Atlantic salmon (*Salmo salar* L.) macrophages treated with yeast β-glucan and bacterial lipopolysaccharide. *Fish & Shellfish Immunology* 11:23–37

Prakash P and Gupta N .(2005). Therapeutic uses of Ocimum sanctum Linn with a note on eugenol and its pharmacological actions: a short review. Indian J Physiol Pharmacol. 2005 Apr; 49 (2):125-31.

Pszczola D.E. (2002). Antimicrobials: setting up additional hurdles to ensure food safety. Food and Technology 56, 99–107.

Reuter H.D., Koch H.P. & Lawson L.D. (1996). Therapeutic effects and applications of garlic and its preparations. In: Garlic, the Science and Therapeutic Application of Allium sativum L. and Related Species (ed. by H.P. Koch & L.D. Lawson), pp. 135–212. Williams and Wilkins, Baltimore.

Robertsen, B. (1999). Modulation of the non-specific defence of fish by structurally conserved microbial polymers. *Fish & Shellfish Immunology* 9:269–290.

Safina, M. M; Christopher M. A; Erick, O. O; Reuben, O; Harrison C. K and Jonathan, M M (2013). Haematological Response of African Catfish (Clarias gariepinus Burchell 1822) Fingerlings Exposed to Different Concentrations of Tobacco (Nicotiana tobaccum) Leaf Dust. ISRN Zoology, Volume 2013 (2013), Article ID 492613, 7 pages.

Sahoo, P.K. and Mukherjee, S. C. (2003). Immunomodulation by dietary vitamin C in healthy and aflatoxin B1-induced immunocompromised rohu (*Labeo rohita*). Comp Immunol Microbiol Infect Dis 26:65-76.

Sahu S., Das B.K., Mishra B.K., Pradhan J. & Sarangi N. (2007). Effects of Allium sativum on the immunity and survival of Labeo rohita infected with Aeromonas hydrophila. Journal of Applied Ichthyology 23, 80–86.

Sakai, M., K. Taniguchi, K. Mamoto, H. Ogawa and M. Tabata (2001). Immunostimulant effect of nucleotide isolated from yeast RNA on carp (*Cyprinus carpio* L.). *Journal of Fish Diseases* 24:433-438.

Sakai, M. (1999). Current research status of fish immunostimulants. *Aquaculture* 172:63-92.

Siwicki A.K. (1989). Immunostimulating influence of levamisole on non-specific immunity in carp (Cyprinus carpio). Developmental and Comparative Immunology 13, 87–91.

USDA (2007). Garlic goodness best released with a crush. United States Department of Agriculture, Agricultural Research Services. http://www.ars.usda.gov/is/pr/2007/070918.

Villamil, L., Figueras, A and Novoa., B (2003). Immunomodulatory effects of nisin in turbot (*Scopthalmus maximus* L.). *Fish & Shellfish Immunology* 14:157–169.

Yano, T. (1992). Assay of hemolytic complement activity. In: Stolen, J.S.; Fletcher, T.C.; Anderson, D.P.; Haattari, S.L.; Rowley, A.F., (eds.). Techniques in fish immunology. Poland: SOS Publications 131–141.