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Immunostimulatory Effect of Phoenix Dactylifera Supplemented Diet on *Aeromonas hydrophila* Infected *Clarias gariepinus*

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Abstract

Background: *Phoenix dactylifera* fruit has been widely reported for its effective anti-inflammatory properties. Therefore, the effect of *P. dactylifera* on immune responses was evaluated in *Aeromonas hydrophila* infected *Clarias gariepinus*.

Methods: *P. dactylifera* fruit powder was used as feed inclusion at 0 (control), 0.5, 1.0 and 2.0% concentrations to the pelleted fish diet of *C. gariepinus* juveniles. A total of 75 fish were divided into four groups (n=15) . The fish were fed with the formulated feed twice a day (08:00 h and 17:00 h), and terminated 24 h before the commencement of the microbial inoculation with inactivated *Aeromonas hydrophila* bacteria. The fish were later challenged with active *Aeromonas hydrophila* after seven weeks of experimental feeding. Blood samples were collected from fish while haemagglutination and phagocytic assays were used to determine the antibody titres and phagocytic action of the immune cells, respectively. Then, the micronucleus assay was used to determine the chromosomal damage effect of *Aeromonas hydrophila* pathogen on exposed *C. gariepinus*.

Results: After the challenge, the groups fed with 0.5 and 2.0% date showed significantly ($p<0.05$) elevated level of immune responses with antibody titres of 231.49±77.06 and 257.18±51.37, and phagocytic activity values of 0.07±0.01 and 0.04±0.00, respectively. The clastogenic effect of *A. hydrophila* on theperipheral blood of infected *C. gariepinus* was observed, this was significantly reduced in 2% *P. dactylifera* fed group. Also, increase white blood cell count was observed in *the C. gariepinus* group fed with 0.5% date supplement. A higher lymphoid organ index was also observed in fish fed with the supplemented diet, as compared to the control.

Conclusions: These results indicate that *P. dactylifera* fruit powder enriched diet showed an immune-boosting and reduction of the DNA damaging effect in bacteria-infected *C. gariepinus*

Keywords: *Aeromonas hydrophila*, Antibody titre, *Clarias gariepinus, Phoenix dactylifera,* Vaccination

1. INTRODUCTION

The health of an organism largely depends on the competency of its immunity. The immune system has been shown to play a pivotal role in the aetiology and pathophysiological mechanisms underlying many diseases and so, over the years, have become a critical aspect of scientific research [1]. Kidney, thymus, spleen, liver, and mucosa-associated lymphoid tissues (MALT) such as the gut, gills and heart are important immune competent organs of fish [2]. These organs are part of the fish immune system, which constantly ensure that invading pathogens are destroyed [3]. During invasions, numbers of phagocytic cell such as monocytes, lymphocytes and neutrophils may increase or decrease according to the health status of the fish[4].

Heavy mortalities have been recorded in both cultured and wild fish as a result of bacterial disease infestation, most of which are naturally occurring saprophytes[5] *A. hydrophila* is a motile group of gram negative bacteria belonging to the family the Aeromonadaceae. They are widely distributed in aquatic as well as terrestrial organisms; grow rapidly in moderate temperature ranging between 20-45^{o}C, which makes fish one of the most vulnerable animals at risk of *A. hydrophila* infection [6]. Also, its highly variable pathogenesis allows *A. hydrophila* to release substantial amount of its extracellular product (ECP) and induce cytotoxic, cytolytic, and haemolytic and enterotoxic properties leading to *aeromoniasis* infection in fishes[7]. *Clarias gariepinus* is a freshwater fish that is widely available as culture fish in Nigeria, as well as throughout tropical Africa [8]. They are one of the culture fish of choice due to their adaptability to varied water conditions and their omnivorous feeding habit [9].

The use of synthetic antibiotics and chemotherapeutics have been touted as a major contributing factors to the growing lists of resistant pathogen and chemical induced environmental pollution. Outcomes of these products when used as vaccines are not always successful and their sustainable use has been reported as a great influence in the development of more pathogen resistant strains of micro-organisms affecting both juvenile and adult fish [10]. The need to curb excessive application of synthetic chemicals in fish culture due to risks caused to humans through consumption of chemical residues in food and antibiotic resistance being passed on to human pathogens, is necessary [11]. Therefore, the use of whole, parts and plant derived compounds (phytochemicals) in fish immunity has become a rapidly growing area of research.

Dietary use of *Phoenix dactylifera* (commonly known as date) has shown that it contains vitamins, dietary fibers, reduced fat and proteins that the human body requires. According to Baliga *et al*. [12], consumption of hundred grams of date can provide over 15% recommended daily allowance of the necessary trace elements such as zinc, selenium, copper, sodium, potassium, etc. Date fruit as well as its aqueous extract have been reported for the free radical scavenging activity, anti-mutagenic and anti-inflammatory effects [12]. However, few studies have been carried out with focus on the immunomodulatory activities of date fruit and most of the literature available are basically with the extract and mice as model [13–16], and without clear understanding of the basic mechanism. Therefore, there is need to carry out studies on the immunomodulatory effects of date fruit supplementary diets in *C. gariepinus*.

2. MATERIALS AND METHODS

This research was conducted in the Cell Biology and Genetics Unit laboratory of the Department of Zoology, University of Ibadan (UI), Nigeria. The research was approved by Animal Care and Use Research Ethic Committee (UI- ACUREC) assigned Number is UI-ACUREC/18/0102

2.1 Preparation of *Aeromonas hydrophila* Pathogen and Inoculation Procedure

Aeromonas hydrophila pathogens cultured in nutrient broth following Biller-Takahashi *et al*. [17] was obtained from the Department of Microbiology, UI. Briefly, the bacteria cells obtained were suspended in Phosphate buffer saline. The suspensions were washed three times and spun for 3 minutes at 3000 rpm in sterile PBS. The bacterial cells were then inactivated with 3% formalin treatment for 24 hours at room temperature, washed three times and diluted to 1.5 x 10^{9}cfu ml^{-1}concentration in PBS medium using standard turbidimetric assay according to Biller-Takahashi *et al*. [17]. The effectiveness of the inactivation process was ascertained by culturing the suspension of *A. hydrophila* cells prepared and rate of colony formation compared with untreated cells.

2.2 Experimental Design

Freshly collected date fruits were purchased from Bodija market, sorted, and oven dried at 37^{o}C. The dried pulps were then pulverized into powder, dry-mixed at 0, 0.5, 1.0 and 2.0 percent concentrations with a formulated fish diet (3 mm pellets) as presented in Table 1.

Table 1. Formulation of Experimental Diets

Ingredients (g)	Graded levels of P. dactylifera Fruit Powder (%)			
	0	0.5	1.0	2.0
Soya bean meal	25	25	25	25
Date powder	0	0.5	1.0	2.0
Fish meal	25	25	25	25
Rice bran	17.5	17	16	15.5
Wheat bran	17.5	17.5	17.5	17.5
Bone meal	3.0	3.0	3.0	3.0
G. N. C	10	10	10	10
Premixes	1.0	1.0	1.0	1.0
Wheat flour (binder)	1.0	1.0	1.0	1.0
Total	100	100	100	100

Clarias gariepinus juveniles acquired from the Fishery Department of Oyo State Ministry of Agriculture, Ibadan were acclimatized for 21 days in 50 litres white transparent plastic tanks. A total of sixty *C. gariepinus* was divided into four (4) groups (A-D) consisting of 15 fish/50litre tank with respect to Date supplemented (DS) feed concentrations, and inoculation, as follows: group A, fed on 0% DS; group B, 0.5% DS; group C, fed on 1.0% DS, and; group D, 2.0% DS. All the fish received 0.1ml *A. hydrophila* pathogen via Intraperitoneal injection[17]. A separate group (NV) (n=15), was not vaccinated to compare genetic damaging effect and phagocytic indices. Experimental activities and procedures are presented in Table 2.

Haematological analysis

Three fish from each group were sampled for blood collection 2days after Active *A hydrophila* bacterial challenge. Blood was collected from the caudal vein into an EDTA lithium tubes. Parameters such as red blood cell count (RBC), platelet count and total white blood cell count (WBC) were completed using the Neubauer haemocytometer[18]. The packed cell volume (PCV) and haemoglobin (Hb) concentration values were determined by the microhaematocrit capillary tube and cyanomethaemoglobin methods as described by Hesser [19]. The differential leukocyte count (DLC) for lymphocytes, monocytes, eosinophil, neutrophil and basophil was determined using standard procedure[20].

Serum Agglutinating Antibody Titre

The heamaglutination assay was done as described by Afolayan *et al*.[21], using 96 well microtiter plates with round bottom wells. Briefly, a dilution of 1:1 (50µL of phosphate buffer: 50 mL of serum) was prepared in the first rows of wells and subsequently followed by two-fold serial dilutions, which was completed by adding 50 µL of diluted serum into the remaining wells with 50µL of phosphate buffer. Subsequently, 50 µL of formalin killed *A. hydrophila* (1 × 109cfu) suspension was added to each well after which the microtiter plate was covered with plastic film and incubated at room temperature. Plates were read after 3 and 18 hours. The agglutination

Table 2: Experimental Design for Immune Response

S/No	Duration	Activity	Treatment/Procedures
1	Day 0	Vaccination with inactivated bacteria	Intraperitoneal injection of 0.1ml of 1.5 x 10^9cfu ml^{-1} concentrated formalin killed Aeromonas hydrophila pathogen in PBS medium
2	Day 0-54	Experimental Feeding	Group NV – 0% date supplemented feed Group A – 0% date supplemented feed Group B – 0.5% date supplemented feed Group C – 1.0% date supplement feed Group D – 2.0% date supplement feed
3	Day 13	Blood collection 1	0.3-0.5ml of peripheral blood samples collected from the caudal vein to obtain serum for primary antibody titre determination.
4	Day 30	Repeat Vaccination (inactivated pathogen)	Same as day 0
5	Day 39	Blood collection 2	Same as day 13; obtain for secondary antibody titre.
6	Day 50	Challenge (active pathogen)	Intraperitoneal injection of 0.1mlof 1.5 x 10^9cfu ml^{-1} concentrated live *Aeromonas hydrophila* pathogen in PBS medium
7	Day 52	Blood collection 3 and slides preparation	Blood collected for post-challenged antibody titre determination, slide preparation for phagocytosis and micronucleus evaluation
8	Day 52	Sacrifice	Dissection and collection of organs; liver, gill, heart and kidney.
9	Day 52-80	Post challenge observation	Observation for physiological and behavioural change

completion point was taken as the last serum dilution where at least 50% haemagglutination was visible and antibody titre was recorded as log2 of the reciprocal of the dilution factors.

Determination of Phagocytic Activity and Index

Phagocytic cells were identified following a modified method described by Prabu *et al*. [22]. 100µL of *A. hydrophila* (1×10^9cfu) cells suspended in PBS (pH 7.2) was added to each of the five selected wells that already contained 100µL of blood samples which was then gently shaken to obtain a homogenous mixture and incubated for 20 minutes at room temperature. Smears were prepared using 5µL of the incubated mixture. Slides were air dried, fixed with 95% ethanol for 5 min and stained with Giemsa solution for 30 minutes.

A total of one hundred monocytes and neutrophils; numbers of phagocytizing cells as well as the amount of bacteria engulfed by the phagocyte were counted and recorded, from each smear.

Determination of *A. hydrophila* Genomic Damage Induction

Three fish were selected from each of the treatment groups. From each fish, two slides were prepared. The smears were fixed for 20 minutes in absolute methanol, air-dried and then subjected to 10% Giemsa solution for 30 minutes. Any small, circular or ovoid, non-refractile chromatin bodies showing a similar stain pattern as the central nucleus was referred to as Micro-Nucleus (MN) [23]. Nuclear Aberrations (NA) were categorized following Carrasco *et al*. (1990) description. Briefly, cells with two nuclei were recorded as binucleated. From each group, 1000 cells were scored under 1000× magnification. Frequencies of MN and NA were expressed per 1000 cells (%). Organ coefficient (relative organ weight) and other growth parameters were calculated in accordance with Kumari and Sahoo [24], as follows:

$$\text{Feed consumption} = \text{Unconsumed dried feed} - \text{Total weight of feed offered}$$

$$\text{Weight gain (\%)} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}} X \frac{100}{1}$$

$$\text{Feed conversion ratio (g)} = \frac{\text{Feed intake (g)}}{\text{Wet weight gain (g)}}$$

$$\text{Protein efficiency ratio (g)} = \frac{\text{Wet weight gain (g)}}{\text{Protein intake (g)}}$$

$$\text{Specific growth rate (\%)} = \frac{\text{Log e final (g)} - \text{Log e initial weight (g)}}{\text{Number of days}} X \frac{100}{1}$$

$$\text{Organ coefficient (\%)} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of animal (g)}} X \frac{100}{1}$$

2.5 Statistical Analysis

Numerical values were expressed as a Mean ± Standard error of mean (M±SEM). Statistical analysis of the means of expression data among the four treatment groups were performed using one-way Analysis of variance (ANOVA) followed by Duncan's Multiple Range test. Analyses were performed using Microsoft Office Excel v 17.1 for Windows 10, SPSS 21.0.0 (Chicago, USA) for Windows and Graphpad Prism version 5.0 1. The effect of *P. dactylifera* fruit powder on immune response in *C. gariepinus* infected with *A. hydrophila* pathogen is presented as antibody titre ($\log_{2x+1}$).

3. RESULTS

3.1 Effect of *P. dactylifera* Fruit Powder on Growth and Feed Efficiency

Clarias gariepinus juveniles fed on graded levels of test diets that contain 0.5%, 1.0% and 2% concentrations of *P. dactylifera* as feed additive for 8 weeks showed no significant difference ($p<0.05$) on feed consumption, feed conversion and protein efficiency ratio when compared to the control during the feeding trial (Table 3), however, 1.0% date supplement showed higher mean values for feed consumption (29.89), feed conversion (1.57) and protein efficiency ratio (0.47).

Table 3: Effect of *P. dactylifera* Fruit Powder on Weight Gain in *C. gariepinus* Juveniles.

Treatment	Initial	Final	Weight Change
0% date supplement (control)	15.84±0.67	17.76±0.36^{abc}	1.92±0.36^{ab}
0.5% date supplement	14.82±0.67	16.87±0.34^{ab}	2.05±0.34^{ab}
1.0% date supplement	15.83±0.80	18.95±0.48^{b}	3.12±0.48^{b}
2.0% date supplement	14.83±0.56	18.14±0.63^{bc}	3.31±0.36^{b}

Mean (±SEM) values with different alphabet are significantly different at ($p<0.05$) as determined by Duncan's tests.

Effect of *P. dactylifera* fruit powder on weight gain (Table 3) showed that the control (1.92±0.36) was significantly lower than 1.0% (3.12±0.48) and 2.0% (3.31±0.36) fish groups. Also, result shown in Table 4 revealed an obvious increase in growth rate, specific

growth rate and length increase in 1.0% (3.12±0.48, 16.37±5.07 and 0.15±0.02) and 2.0% (3.31±0.63, 22.32±4.28 and 0.16±0.03) groups, relative to control (1.92±0.36, 12.12±2.33 and 0.10±0.02).

Table 4: Effect of *P. dactylifera* Fruit Powder on Growth Indices of *C. gariepinus* Juveniles

Treatment	Growth Rate	Specific Growth Rate	Length Increase	Specific Length Increase
0% date supplement (control)	1.92± 0.36^{ab}	12.12± 2.33^{ab}	0.10± 0.02^{ab}	7.13± 3.01
0.5% date supplement	2.05± 0.35^{ab}	13.83± 2.34^{ab}	0.11± 0.02^{ab}	4.92± 2.10
1.0% date supplement	3.12± 0.48^{b}	16.37± 5.07^{b}	0.15± 0.02^{b}	7.19± 4.54
2.0% date supplement	3.31± 0.63^{b}	22.32± 4.28^{b}	0.16± 0.03^{b}	11.65± 3.96

Mean (±SEM) values with different alphabet are significantly different at ($p<0.05$) as determined by Duncan's tests.

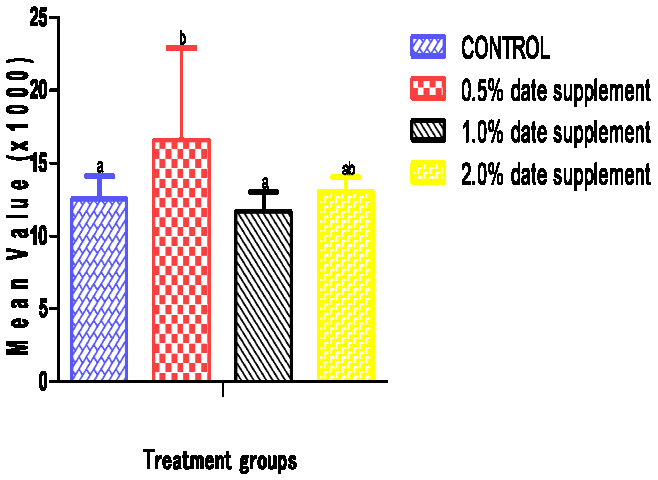

Figure 1. Effect of Feed Supplemented with *P. dactylifera* Fruit Powder on White Blood Cells (WBC) of *C. gariepinus* juveniles.
Mean (±SEM) values with different alphabet significantly different ($p<0.05$) as determined by Duncan's tests.

3.2 Effect of *P. dactylifera* Fruit Powder on Haematological Parameters

The haematological parameters are summarized in Table 5. Results showed no significant variations ($p<0.05$) in packed cell volume (PCV), red blood cell counts (RBCs), and haemoglobin (HB) values among all inclusion levels or in relation to the control groups. Results in Figure 2 shows a significant increase in white blood cell (WBC) counts for 0.5% (16.56±6.33) when compared to the control group (12.50±1.62). It is interesting to note that platelet cells for the 2.0% (9.17±1.78) date was lower compared to the control (11.03±1.60).

3.3 Effect of *P. dactylifera* Fruit Powder on Organ Index

The effect of date fruit powder on lymphoid organs relative to net body weight is shown in Table 6. Significantly higher index was recorded for liver in 0.5% (1.52±0.31) and 2.0% (1.70±0.06) treatment groups than 1.0% date supplement (1.25±0.08) and control (1.25±0.32) groups. While, 0.5% (0.37±0.02) and 1.0% (0.34±0.05) treatment

Table 5: Effect of *P. dactylifera* Fruit Powder on Haematological Parameters

Treatments	Haematological Parameters									
	PCV	PLT (10^4)	RBC	HB	WBC (10^3)	LYM	NUT	MON	EO	BA
0% date supplement (control)	18.67± 1.86^{a}	11.03± 1.60^{a}	1.24± 0.06	6.10± 0.70	12.50± 1.62^{a}	63.33± 0.88	27.00± 0.58	3.33± 0.88	5.00± 1.00	0.67± 0.33
0.5% date supplement	19.67± 1.20^{a}	10.23± 2.05^{a}	1.29± 0.08	6.17± 0.52	16.56± 6.33^{b}	55.00± 3.21	37.00± 4.04	4.00± 0.58	4.00± 1.15	0.00± 0.00
1.0% date supplement	17.33± 0.88^{a}	11.36± 1.34^{a}	1.13± 0.05	4.83± 0.38	11.67± 1.35^{a}	56.00± 3.06	37.67± 2.84	2.67± 0.33	4.00± 0.58	0.33± 0.33
2.0% date supplement	17.33± 0.88^{a}	9.17± 1.78^{a}	1.17± 0.17	5.67± 0.03	13.05± 0.99^{ab}	60.33± 2.60	31.33± 3.48	2.67± 0.67	5.33± 1.20	0.33± 0.33

Mean (±SEM) values with different alphabet are significantly different at ($p<0.05$) as determined by Duncan's test.

Parameters: PCV = Packed Cell Volume, RBC = Red Blood cell Count, HB = Haemoglobin, WBC = White Blood cell Count, LYM = Lymphocytes, NUT = Neutrophil, MON = Monocyte, EO = Eosinophil, BA = Basophil, PLT = Platelets.

Table 6. Effect of *P. dactylifera* Fruit Powder on Organ Indices

Treatments	Net Body Weight Change	Organ Weight and index							
		Liver		Kidney		Gills		Heart	
		Absolute Weight	Organ Index	Absolute Weight	Organ Index	Absolute Weight	Organ Index	Absolute Weight	Organ Index
0% date supplement (control)	27.80±2.46	0.37±0.11	1.25±0.32^{a}	0.07±0.01^{a}	0.25±0.05^{ab}	0.90±0.17	3.16±0.39	0.03±0.00	0.11±0.00
0.5% date supplement	27.23±3.08	0.43±0.13	1.52±0.31^{ab}	0.09±0.01^{ab}	0.37±0.02^{bc}	0.78±0.12	2.86±0.31	0.04±0.01	0.14±0.01
1.0% date supplement	25.97±2.67	0.33±0.05	1.25±0.08^{a}	0.09±0.01^{ab}	0.34±0.05^{abc}	0.94±0.07	3.66±0.78	0.03±0.00	0.10±0.00
2.0% date supplement	29.50±1.76	0.50±0.03	1.70±0.06^{ab}	0.06±0.00^{a}	0.21±0.02^{a}	0.87±0.08	2.98±0.43	0.13±0.09	0.44±0.33

Mean (±SEM) values with different alphabet are significantly different at ($p<0.05$) as determined by Duncan's tests

groups were observed to be significantly higher than the 2.0% fish group (0.21±0.02) and control (0.25±0.05) groups for the kidney.

Effect of *P. dactylifera* Fruit Powder on Immune Response

Figure 2 presents the effect of P. dactylifera fruit powder on immune response in C. gariepinus infected with A. hydrophila pathogen. There was a progressive increase observed for titres values (primary and secondary antibody) obtained across all treatment groups. The primary antibody titres obtained were higher for 1.0% (25.98±6.42) and 2.0% (25.98±6.42) fish groups; 0.5% (141.58±84.22) and 1% (103.05±25.68); while the secondary antibody titres revealed, 0.5% (231.50±77.06) and 2.0% (257.18±51.37) experiments compared to control (16.35±3.21 and 51.67±12.84, respectively).

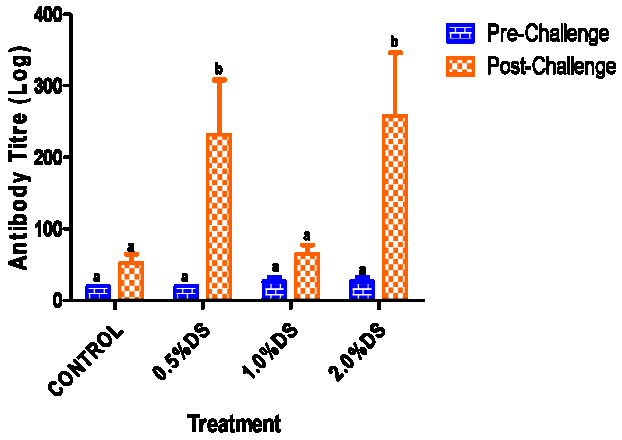

Figure 3. Pre-Challenge and Post-Challenge Antibody Titre.
Mean (±SEM) values with different alphabet are significantly different ($p<0.05$) as determined by Duncan's test. 0.5% DS = 0.5% Date Supplement, 1.0% DS = 1.0% Date Supplement, 2.0% DS = 2.0% Date Supplement

3.5 Effect of *P. dactylifera* on the Genetic Damaging Impact of A. *hydrophila pathogen*

The frequencies of MN, NA, and Total aberration (TA) obtained for fish infected with *A. hydrophila*as presented in Table 8 showed contrasting trends in all of the highlighted abnormalities as reported for the post-challenge and pre-challenge analysis. There was a dose-dependent increase in abnormalities for the pre-challenge, while an alternate reduction in abnormalities was observed across all the treatment groups for the post-challenge analysis.

3.6 Phagocytic activity and index

Table 9 presents the summary of phagocytic activity and index. The serum phagocytic activity exhibited a reducing trend and increasing trend for phagocytic index, in relation to the increase in date fruit powder incorporated diet. A clear significant difference ($p<0.05$) of phagocytic activity was observed in the 0.5% (0.07±0.01) fish group and that of phagocytic index in the 1.0% (2.68±0.25) fish group as compared to the non-vaccinated fish group (0.03±0.01 and 1.22±0.12, respectively) (Figure 6).

4 DISCUSSION

Aeromonas hydrophila is a significant cause of bacterial infections in fishes, including catfish (*Clarias gariepinus)*, carp (*C. carpio*), largemouth bass (*Micropterus*

Table 7. Effect of *P. dactylifera* Fruit Powder on Immune Response

Treatment	Pre-challenged Antibody Titre		Post-challenged Antibody Titre
	Primary	Secondary	Challenge
No vaccination (NV)	21.17±9.76[a]	25.98±6.42[a]	19.94±5.61[a]
0% date supplement (Control)	16.35±3.21[a]	51.67±12.84[a]	51.67±12.84[a]
0.5% date supplement	19.56±0.00[a]	141.58±84.22[b]	231.49±77.06[b]
1.0% date supplement	25.98±6.42[a]	103.05±25.68[b]	64.52±12.84[a]
2.0% date supplement	25.98±6.42[a]	38.83±0.00[a]	257.18±51.37[b]

Mean (±SEM) values with different alphabet are significantly different at ($p<0.05$) as determined by Duncan's test.

Table 8. Effect of *P. dactylifera* on the Genetic Damaging Impact of A. *hydrophila* pathogen in *C. gariepinus*

Treatment groups	Cellular Abnormality					
	Pre-Challenge			Post-Challenge		
	Micronucleus	Nuclear aber-ration	Total aberra-tion	Micronucleus	Nuclear aber-ration	Total aberration
No vaccination (NV)	4.00±1.63[a]	14.00±4.76[a]	18.00±6.00[ab]	40.00±6.73[a]	6.00±2.58[ab]	46.00±6.21[b]
0% date supplement (control)	10.00±4.76[a]	15.00±4.12[ab]	25.00±7.72[ab]	45.00±11.94[b]	11.00±2.51[b]	56.00±4.32[b]
0.5% date supplement	5.00±1.92[a]	5.00±1.91[a]	10.00±2.58[a]	12.00±3.26[a]	0.00±0.00[a]	12.00±1.63[a]
1.0% date supplement	8.00±2.83[a]	23.00±3.41[b]	31.00±2.51[b]	8.00±2.82[a]	3.00±1.91[a]	11.00±3.78[a]
2.0% date supplement	22.00±4.78[b]	26.00±7.02[b]	47.00±3.41[c]	11.00±1.91[a]	2.00±1.15[a]	13.00±2.51[a]

Mean (±SEM) values with different alphabet are significantly different at ($p<0.05$) as determined by Duncan's tests.

salmoides), Nile tilapia (*O. niloticus*) and striped bass (*Morone saxatilis*) [6]. Medicinal plants have been suggested as a sustainable and natural growth-promoting alternative in fish [25]. In the present study, inclusion of date fruit powder especially at 2% showed significant effect on weight and length increase when compared to control. The positive trend of immunostimulation demonstrated by fish on date enriched diet is an indication that date fruit possesses the ability to aid digestion of feed for effective utilization without detrimental effects [12]. The current result agrees with previous findings, where *P. dactylifera* fruit exhibited growth-promoting effects [26,27].

The measurement of organ weights such as kidney, liver, gills and heart in relation to body weight can be directly associated with some environmental changes that act on the general status of health of the fish. In the present study fish fed with feed supplemented date fruit at different concentrations showed a slight significant increase in the index of all selected organs, beside the gills, when compared to control (Table 6). This may be an indication that the stress induced by *A. hydrophila* pathogens injected in the fish has over time induced morphological changes in the selected organs. This is, however, in contrast to reports of Ensibi *et al*. [28], Jordaan *et al*.[29] and Nwani *et al*.[30] that no significant effect was detected in fish organs exposed to pyrethroid, organophosphate and paraquat toxicants, respectively, when they were compared to control. Exposure to the bacterial pathogen may have altered some regulatory function of the immune system, thus, contribute to the observed physiological changes in the selected organs.

Different species of teleost including *Clarias* fish produce specific IgM-type natural antibodies against various antigens [31]. Significantly higher agglutination titre observed in the 2.0% and 0.5% treatment groups as compared to the control could be an indication that *P. dactylifera* fruit possess active immunostimulatory compounds. Selenium, carotenoids, quercetin, kaempferol and isorhamnetin are among the bioactive compounds present

in date fruit [32] and that these compounds possess immunostimulatory effects, which may as well be accountable for the perceived effects in the current study. The present finding is in line with report of Yeasmin *et al*. [33] in Nile tilapia (*O. niloticus*) [34] where 2.0% *L. indica* and fenugreek seed supplemented diets fed fish showed the highest immune response against *A. hydrophila.* This study result agrees with the finding of Ji *et al*. [34] where 0.5% dietary supplementation of powdered fruit of *Massa medicata* fed to red sea bream, *Pargus major* for 12 weeks observably activates the immune system of the fish. Perveen *et al*. [35], in their study also showed similar antibacterial action of *P. dactylifera* leaf and pit extracts against *Streptococcus pyogenes*.

In this experiment, *C. gariepinus* juvenile fed with date supplement showed a higher immune response than the control that was fed without date. The positive trend observed across all groups follows typical immunoglobulin response after primary and challenge immunization [36]. The post-challenge response observed may be as a result of the large numbers of memory cells produced after primary (pre-challenge) B-cell activation [37].

Phagocytes act as cellular scavengers and so, constitute an important part of the host defence system against infectious diseases [38]. Phagocytic ability of leukocytes such as monocyte against xenobiotic amoeboid movement or phagocytosis by neutrophil are well established [4]. Thus, higher amount of leukocytes comprising neutrophil, monocyte and lymphocyte observed in fish groups that were fed with date at 0.5% and 1.0% date supplement corroborates with findings of Thanomsit *et al*. [4].

An increase in phagocytic activity indicated the significant role of *P. dactylifera* enhancing the immune response. Similar finding has been reported in rainbow trout fed with *A. sativum*, *L. perennis, M. indica,* and *U. dioica* against *A. hydrophila* infection [33]. The free radical scavenging and antioxidant activities of lignin, selenium and phenolic compounds present in the date fruit may be responsible for the observed antimicrobial and phagocytic activity [32].

Haematology indices are key assumptions that can be used to evaluate the effects of dietary treatments on animals [4]. Blood parameters assessed, such as packed cell volume (PCV), haemoglobin (HB), and red blood cell counts (RBC's), although not statistically significant, could serve as an indication that *P. dactylifera* can be incorporated in diet to prevent anaemic condition. White blood cells (WBC) that circulate the blood in search of foreign particles are believed to increase quickly when infections occur, therefore the increase in number of WBC observed in infected fish (Figure 1) may serve as a defensive barrier against pathogenic attack [39]. The increased WBC counts recorded in the present study specify the immunostimulatory effect and anti-bacterial potential of *P. dactylifera* fruit [12].

According to Talpur *et al.* [39], when fish stop feeding as a result of disease or stress, a possible indication of this is low haematocrit (PCV %) level. The decreased HB content in the fish (Table 5), may point to the fact that the fish were stressed as a result of the *A. hydrophila* infection. The present study agrees with Mason [40] that reported similar observation when *C. gariepinus* was subjected to a sub-lethal concentration of formalinas well as well as studies of Thanomsit *et al*. [4] on cytological alteration of leucocytes and Kumolosasi *et al.* [41] on immunostimulant activity of *Magnifera indica* leaf and *Curcuma domestica* rhizome in mice.

Micronucleus analysis has over time been used as a successful probe for detecting genomic damage in aquatic species [42]. The present study examines the level of *A. hydrophila* induced toxicity on the peripheral erythrocytes of *C. gariepinus* juvenile fed with varying levels of test diet and evaluated using the piscine MN assay. DNA damage arising from micronuclei formation likely contributes to immune activation [43,44]. Therefore, the significant increase in 2.0% group may be due to the level of immune reaction the test feed was able to stimulate in the pre-challenge. Furthermore, the contrasting reduction in significant difference observed in this same group for the post-challenge test (Table 8) might have arisen due to memory cells that aid rapid stimulation of immune response.

These results also showed that there is significant stimulation of genotoxic stress, which may be as a result of the release of extracellular product by *A. hydrophila* cells after injection[7]. The outcome in the current study is also similar to reports in previous studies [23,42]. The assertions that extracellular product can induce MN and NA observed in the peripheral erythrocytes of the *C. gariepinus* juveniles is corroborated by findings of Bartsch *et al*. [43], Gekara [45], and Harding *et al*. [44].

The effects of selenium in low concentration against carcinogens and mutagens as well as anthocyanins, proanthocyanidins, phenolics, and β- carotene against mutagens have been reported[12]. Therefore, the cumulative

effect of these components in the test substance may have been accountable for the observed reduction in effect of *A. hydrophila* pathogen after challenge.

In conclusion, the present study has shown that date fruit powder included as dietary supplement enhanced growth rate, immune response, phagocytic activity and phagocytic indexes which have collectively contributed to observed resistance against *A. hydrophila* pathogen in treated *C. gariepinus*. This study further demonstrated that *P. dactylifera* fruit powder as a food additive for *C. gariepinus* reduces cellular damage due to active *A. hydrophila* pathogens.

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Authors' contributions

TOO, Collected data, contributed to data, performed data analysis and wrote the paper. **OSA**, contributed to data and analysis. **FIDA**, conceived and designed the study , contributed to data and analysis and wrote the paper.

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