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**Abstract:** This study assessed the effects of hyperthermia –induced stress on survival and expression of heat shock protein in African catfish, *Clarias gariepinus* fingerlings fed aqueous extract from *Moringa oleifera* leaf. Fish were graded by size and groups of 15 fish of 10.00 ± 0.10g and then stocked into glass tanks of 60cm ×45cm×45cm dimension in triplicate. They were fed diets representing different supplementation levels of *Moringa oleifera* leaf extract. The graded levels of *M. oleifera* leaf extract were 0.00g (control), 0.05g, 0.10g, 0.15g, 0.20g, 0.25g per 100g. Six isonitrogenous and isocaloric diets were formulated to meet the requirements of 40% crude protein for *C. gariepinus* fingerlings. After six weeks of the feeding trial, fish previously fed each experimental diet were exposed to hyperthermia – induced stress. Water temperature was maintained at 39±1.30°C throughout the hyperthermia – induced stress period. After hyperthermia – induced stress, fish from each dietary treatment was placed into the culture system and fed their respective diets at 5% body weight twice daily. Mortality was monitored and molecular expression (abundance and diversity) of stress protein genes (heat shock protein, HSP70) in experimental fish was assessed. Result showed that hyperthermia – induced stress mortality were significantly reduced (P< 0.05) by supplementing the fish diets with *M. oleifera* leaf extract. Molecular analyses of the HSP 70 genes showed marked similarity in the banding patterns of fish fed the Moringa leaf supplemented diets in groups MLSC5, MLSC10, MLSC15, MLSC20 treatments with a corresponding increase in fish survival in these treatments. Based on the result of this study, a dose of 0.10g/100g dietary Moringa leaf supplementation was sufficient as a stress reducing agent and improved the survival in African catfish subjected to hyperthermal-induced stress.

**Key words:** *Clarias gariepinus* • Supplementation • Moringa • Hyperthermia • Survival • Heat Shock Proteins Expression

**INTRODUCTION**

Cultured fish are unavoidably exposed to wide ranges of stimuli associated with environmental stress and pathological challenges. All environmental factors can influence the degree to which fish respond to stressors. External factors include acclimation temperature, salinity, time of day, wave length of light and even background color of the tanks. Internal environmental factors, including the fish’s nutritional state and presence of disease may also affect the magnitude of the stress response. There is also increasing concern about the effect of high temperature on aquaculture species due to climate change and aquaculture related heat stress [1 -3]. Primary stress responses in teleost fishes include a number of hormonal changes, particularly those in circulating levels of cortisol and catecholamines [5]. Secondary responses, which may or may not be caused directly by the endocrine response, include measurable changes in blood glucose, lactate or lactic acid, and major ions (e.g., chloride, sodium, and potassium), and tissue levels of glycogen and recently HSPs (Heat Shock Proteins) Tertiary responses, includes changes in growth, disease resistance and behavior.
The measurement of plasma cortisol alone may not necessarily reflect the degree of stress experienced by the fish at that instant but more likely be representative of the extent of the earlier or initial response [2]. Moreover, understanding trends in changes that occur in fish in response to stressors can often provide clues that help relate the physiological responses of individuals with changes in performance that could affect their health and survival [6].

Pathological, transportation and temperature stress, particularly rapid changes in temperature, severely limits the fish’s ability to release antibodies, giving the invader the time to reproduce and overwhelm the fish [7, 8]. Strategies to attenuate stress and stress-related negative responses in aquaculture should be considered. For effective stress management, it is important to study the stress response in fish, its functional role and molecular and cellular basis, how the response can be measured, and whether detection of a stress response provides information relevant to the assessment of the health of fish [10]. The appropriate use and interpretation of physiological markers is particularly important when considering fish [11].

Application of novel technologies integrating research to simultaneously examine genomes and growth should help to better understand the modes of action of phytopgenic compounds such as M. oleifera and subsequently lead to the development of feasible and cost-effective ways to use phytopgenic compounds in the sustainable management of stress in aquaculture. Different parts of Moringa have shown great antioxidant activity as well as immunomodulatory function in animals. Moringa leaves have been reported as a rich source of proteins, antioxidants and minerals. Extracts from the leaves and pods have numerous health benefits and seed extracts have been shown to be antimicrobial [12]. Therefore, due to the multiple role of Moringa oleifera Lam. (Moringaceae) in aquaculture [13] a better understanding of the mechanism through which dietary M. oleifera as a nutritional supplement influences the physiology of fish exposed to stress in aquaculture especially at molecular and systemic levels is necessary. There are abundant information on how aquatic animals respond physiologically to general stress however little information is available on the understanding of how aquatic animals respond to thermal stress at the molecular level [14]. In order to provide a sustainable and environmental friendly solution to the adverse effects of stressors in African catfish, C. gariepinus aquaculture, this study was designed to assess the effects of hyperthermia –induced stress on survival and expression of heat shock proteins in African catfish, Clarias gariepinus fingerlings fed aqueous extract from Moringa oleifera leaf.

MATERIALS AND METHODS

Extraction of Moringa oleifera: The leaves of Moringa oleifera were collected from a farm settlement at Ijare, Ondo State, Nigeria. It was identified and authenticated at the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure. The leaves were destalked, washed and dried in the shade. Moringa oleifera leaves were ground with pestle and mortar, leaves were then extracted according to the modified method of Makanjuola et al. [15] as follows. Five hundred grams of the powdered leaf were soaked in 1.5 liter of warm water (60°C). Each solution was allowed to stand for 24 hours, after which it was sieved with a muslin cloth and filtered using No 1 Whatman filter paper. The filtrate were collected in a beaker and concentrated with the aid of rotary evaporator (Resoana, Germany).

Determination of Main Active Compounds in Moringa Oleifera Leaf Extract: In the present study, analytical High Performance Liquid Chromatography (HPLC) for flavonoids (Quercetin (Q) and Kaempferol (K)) was carried out on an Agilent XDB C18 column (250 mm L × 4.6 mm, 5 µm particle) and an Agilent 1100 instrument (Agilent, Palo Alto, CA, USA). Detection was made at the wavelengths of 320 nm and 250 nm respectively. HPLC conditions were as follows: The analysis was performed at a flow rate 1.5 ml per minute. The UV detector was set at k = 320 and 250 nm. Elution with 0.01 M acetate buffer adjusted to apparent pH 3.3 using glacial acetic acid (Solvent A) and acetonitrile (Solvent B) in a step gradient manner was carried out as follows: 60:40 (For 10 min), then 40:60 (For 5 min) and finally wash with 10:90 (For 10 min). All determination was performed at ambient temperature. The injection volume was 20 µl. Standard solutions of Q and K were prepared in methanol within a concentration range of 0.4–20 µg per ml for each of Q and K. Twenty µl injections were made for each flavonoid concentration. The peak height values were plotted against corresponding absorbence. The contents of the analytic were determined from the corresponding calibration curves. The contents of quercetin (Q) and kaempferol (K) were found to be 0.21 mg /g and 0.14 mg /g respectively.
Table 1: Composition of the experimental diet (g/100g) containing dietary Moringa oleifera for African catfish, Clarias gariepinus fingerlings

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>MLSC0</th>
<th>MLSC5</th>
<th>MLSC10</th>
<th>MLSC15</th>
<th>MLSC20</th>
<th>MLSC25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal (68 % CP)</td>
<td>23.50</td>
<td>23.50</td>
<td>23.50</td>
<td>23.50</td>
<td>23.50</td>
<td>23.50</td>
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<tr>
<td>GNC (48 % CP)</td>
<td>29.00</td>
<td>29.00</td>
<td>29.00</td>
<td>29.00</td>
<td>29.00</td>
<td>29.00</td>
</tr>
<tr>
<td>Soybean meal (42 % CP)</td>
<td>20.50</td>
<td>20.50</td>
<td>20.50</td>
<td>20.50</td>
<td>20.50</td>
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<tr>
<td>Yellow maize</td>
<td>10.50</td>
<td>10.50</td>
<td>10.50</td>
<td>10.50</td>
<td>10.50</td>
<td>10.50</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>7.00</td>
<td>7.00</td>
<td>7.00</td>
<td>7.00</td>
<td>7.00</td>
<td>7.00</td>
</tr>
<tr>
<td>Rice Bran</td>
<td>5.50</td>
<td>5.50</td>
<td>5.50</td>
<td>5.50</td>
<td>5.50</td>
<td>5.50</td>
</tr>
<tr>
<td>Alginate</td>
<td>2.00</td>
<td>1.95</td>
<td>1.90</td>
<td>1.85</td>
<td>1.80</td>
<td>1.75</td>
</tr>
<tr>
<td>Vitamin Mineral mix</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Moringa leaf extract</td>
<td>0.00</td>
<td>0.05</td>
<td>0.10</td>
<td>0.15</td>
<td>0.20</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Composition of vitamin-mineral mix (Aquamix) (Quantity/kg), Vitamin A, 55,00,000 IU; Vitamin D3, 11,00,000 IU; Vitamin B2, 2,000 mg; Vitamin E, 750 mg; Vitamin K, 1,000 mg; Vitamin B6, 1,000 mg; Vitamin B12, 6 mcg; Calcium, 500 mg; Pantothenate, 2,500 mg; Nicotinamide, 10 g; Choline Chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450 L- lysine, 10 g; Selenium, 50 ppm.

Preparation of Experimental Diets: The feed ingredients were purchased at Adedom Feedmill, Ondo road, Akure, Ondo State, Nigeria. Six isonitrogenous and isocaloric diets were formulated to meet the requirements of 40% crude protein (Table 1) for C. gariepinus fingerlings (National Research Council, 2010) using feed formulation software (WinFeed soft 2.0, USA). All dietary ingredients were weighed with a weighing top balance (Metter Toledo, PB8001 London). The ingredients were then ground to a small particle size (Approximately 20 µg). Ingredients including Moringa oleifera extract, vitamin and mineral premix were thoroughly mixed in a Hobbart A-200T mixing machine (Hobbart Ltd London England) to obtain a homogenous mass. Alginate, Laminaria digitata (IGV GmbH, Germany) was added as binder. The resultant mash was pressed without steam through a mincer using 2mm diameter die attached to the Hobbart pelleting machine. Diets were immediately air-dried, after drying the diets were broken up, sieved and stored in air-tight transparent plastic containers, labeled and stored until feeding. Standard and official methods [16] were used to perform the proximate analyses of feed of fish in the study.

The graded levels of M. oleifera leaf extract were 0.00g (control), 0.5g, 0.10g, 0.15g, 0.20g, 0.25g per 100g for each diet denoted as MLSC0, MLSC5, MLSC10, MLSC15, MLSC20 and MLSC25 in diet 1, 2,3,4,5 and 6 respectively for C. gariepinus fingerlings.

Experimental Fish and Feeding Trial: C. gariepinus fingerlings were obtained from the Hatchery unit of the Department of Fisheries and Aquaculture Hatchery, Federal University of Technology Akure, prior to the feeding trial. Fish were graded by size and groups of 15 fish of 10.00± 0.10g per replicate for C. gariepinus were stocked into glass tanks of 60cm ×45cm×45cm dimension. A commercial diet, Nutreco ® (40% crude protein) was fed to all fish during a 2-week conditioning period. Each experimental diet was fed to six replicate groups of fish for 70 days. All groups were fed their respective diets at the same fixed rate (Initially 5% of body weight per day). This rate was adjusted each week. Fish were fed by 0900-1000 and 1700-1800h GMT, for 7 days each week. Growth was monitored weekly by batch weighing of fish from each tank.

Physico-Chemical Water Parameters: Dissolved oxygen was monitored using HANNA 98103SE (HANNA instruments, Rhode Island). Temperature and pH were monitored using YSI-IODO 700 Digital probe (IFI Olsztyn, Poland).

Hyperthermia-Induced Stress: At the end of the feeding trial, 18 fish previously fed each experimental diet from each treatment representing 3 fish per treatment were kept in plastic tanks for hyperthermia-induced stress according to a modified method of Sen et al. [17] using a 2-kW heating rod (Binatone, Japan). The temperature treatments was based on a pilot study where the sub-lethal temperature of < 39°C was recorded for C. gariepinus. The rate of heating ramp was about 3°C/h. Water temperature was maintained at 39 ± 0.5°C throughout the hyperthermia-induced stress period. No fish died during the hyperthermia treatment. Two fish were taken randomly at 2h after exposure from the tanks. Two fish per tank were euthanized by overdose (200 mg/liter of water for 10 min) of tricaine methane sulphonate (MS222; Pharmaq, Fordingbridge, UK). Liver samples were removed by dissection and weighed immediately after hyperthermia-induced stress from fish for further analyses.
Table 2: Sequence and conditions of primers used in this study. PCR of the V3–5 region of the African catfish targeting the 16S rDNA genes was performed using universal primers [18] for African catfish

<table>
<thead>
<tr>
<th>No</th>
<th>Author</th>
<th>Oligo Name</th>
<th>F primer name</th>
<th>R primer name</th>
<th>Sequence (5'-3’)</th>
<th>Amplicon size</th>
<th>Region</th>
<th>GC-contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nguyen, 2015</td>
<td>GUO-PRBA338f</td>
<td>PRUN518r</td>
<td>HSP70-F</td>
<td>TGGAGGAGGGTCTTCTGGAC</td>
<td>434</td>
<td>V5</td>
<td>55%F</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HSP70-R</td>
<td>CACCAAAGAAAACAAACGGACTG</td>
<td></td>
<td></td>
<td>54.7%R</td>
</tr>
</tbody>
</table>

**Molecular Expression (Abundance and Diversity) of Stress Protein Genes (Heat Shock Protein, HSP70) in African Catfish:** Extraction of DNA and PCR-TGGE of fish liver were performed using the Qiagen® blood + tissue through the spin column protocol, the gram positive optimisation was used in this kit to enhance the lysis of cells with complex cell walls. The liver was excised from the fish, homogenized and stored at -80°C to assess differences between the dietary groups. Extracted DNA was checked for quality and quantity by agarose electrophoresis and UV spectrophotometry using Nanophotometer P-class (IMPLEN®, USA).

**Polymerase Chain Reaction (PCR) Conditions:** Primers were sourced from literature and emphasis was laid on primers suitable for Temperature gradient gel electrophoresis (TGGE) (Table 2). PCR of the V3–5 region of the liver 16S rDNA genes was performed using primer 968-f as shown in Table 2. All were obtained from the same reputable supplier (Invitrogen, Germany). For extracted DNA, PCR was performed with a 15-mL reaction mixture containing 7.3mL Mango mix (Qiagen®), 1.5mM MgCl₂, 0.15 mL bovine serum albumin (BSA) which prevents reagents sticking to the tube, 0.75mL forward primer, 0.45mL reverse primer and 1.5mL of template DNA. Pure culture PCR was performed using Pyrogen free DNAse water of 4.9mL. Thermocycling conditions were as follows: initial denaturation at 94°C for 2minutes 30 seconds, followed by 60°C for 30 seconds at -0.7 per cycle for the annealing and elongation was at 72°C for 1min. Denaturation was done again at 94°C for 30 seconds for 15 cycles, then 49°C for 30 seconds and elongation at 72°C for 30 seconds. Final denaturation was done for 18 cycles and final elongation for 5 minutes and cooled down to 4°C.

**TGGE Gels Electrophoresis, Staining and Photographing the Gel:** Control gels and Quick load® DNA ladders (Biolabs, New England) were run on every gel. The condition for TGGE machine was at a constant voltage of 65 V for 16 h., the ramp temperature and increasing rate ramp (output change) was at 0.9°C per hour from 55.6 to 69.6°C using a DCode system (BioRad). Photograph was documented with Gel documentation equipment, Gene genius by Syn gene® and processing was done using gene genius snap 6.0 software.

**Statistical Analysis:** This experiment was designed with a completely randomised design (CRD) to test for significant differences in the mean of treatments. The differences between mean of treatments were considered significant at P = 0.05 by one way analysis of variance (ANOVA) using Statistica® software. Follow-up procedures were performed where significant differences occurred in the means using Tukey test. Photographed gel after TGGE was processed for gel diversity and migration pattern using GelAnalyzer 2010a®, gel electrophoresis image analysis software. Minitab 18® statistical software was used to plot the web – profile radar plot for the diversity of the TGGE gels.

**RESULTS**

**Determination of the Concentration of the Main Active Compounds in Aqueous Extracts of M. Oleifera Leaf:** The contents of the analytic were determined from the corresponding calibration curves using HPLC chromatograph of a model mixture of quercetin and kaempferol. The contents of quercetin (Q) and kaempferol (K) were found to be 0.21 mg /g and 0.14 mg /g, respectively corresponding to absorbence of the model mixture.

**Survival of Clarias Gariepinus Fed the Experimental Diets:** Mortality was recorded in all the dietary treatments. However, significantly higher survival (ANOVA; F₁,₁₂ = 1609.58, P= 0.001) was found in fish fed MLSC10 and MLSC15 diets as 98.00 and 97.60 % respectively at the end of the feeding trial (Figure 1).

**Diversity of the Heat Shock Protein 70 (HSP 70) genes and PCR-TGGE of the liver of Clarias gariepinus Fed the Experimental Diets:** Molecular analyses using the PRBA338f primer with the aid of TGGE gel showed marked similarity in the banding patterns fish fed the Moringa leaf supplemented diets in groups MLSC5, MLSC10, MLSC15,
Fig. 1: Cumulative survival of *C. gariepinus* fed the experimental diets for 10 weeks. Values in each row with different superscripts are significantly different (p < 0.05) by using ANOVA post hoc (Tukey test).

Fig. 2: PCR-TGGE gels showing diversity of the HSPs from the PCR-TGGE fingerprints gel within the *C. gariepinus* liver after the feeding trials. Lane 1 = Ladder (Arbitrary markers).

MLSC20 treatments (Figure 2). Each band represents one gene of HSP 70; the ladders are showed on the first well of the band in the TGGE gel. However, dietary effects were observed in the banding patterns, with *M. Oleifera* based diets in fish fed the MLSC10 diets showing the highest expression of the HSP genes on the gel (Figure 3). Fish from Moring supplemented treatments showed an increase in the expression of the HSP genes on the banding profile compared with the control.

**Similarity and richness of the Heat Shock Protein 70 (HSP 70) genes and PCR-TGGE of the liver of *Clarias Gariepinus* Fed the Experimental Diets:** A web-filled radar graph representing the similarity and richness of the
HSPs from the PCR-TGGE fingerprints gel within the *C. gariepinus* liver is presented in Figure 3. There were differences in the distribution of the banding profile of the HSP 70 in the liver of fish used in this study. The fingerprints gel showed that the control significantly displayed lower diversity in the banding profile of the HSP 70 genes in *C. gariepinus* after exposure to *A. hydrophila* and hyperthermal –induced stress. In contrast, the MLSC10, MLSC15 and MLSC20 treatments were the most closely related and clustered into a group distinctly higher expression of the HSP genes compared to the profiles of the control, MLSC5 and MLSC25 groups.

**DISCUSSION**

In the present study, a tendency in fish fed the Moringa leaf -supplemented diets towards a better survival compared with the control group was observed, these differences were also statistically significant. The reason was attributed to the role of the plant in stimulating the immune system by influencing several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability and ultimately enhancing the disease resistance and growth performance of the fish [19]. Flavonoids like quercetine and kaempferol, which was found to have high absorbence in the feed used in this study have been reported to have efficient radical scavenging activity which inhibits the enzymes involved in reactive oxygen species (ROS) generation, that is, microsomal monoxygenase, glutathione S-transferase, mitochondrial succinoxidase and NADH oxidase [20].

In the present study, fish in MLSC10, MLSC15 and MLSC20 groups exhibited the highest post-challenge survival (98.0 %), (98.7%) and (97.6%) respectively. However, the highest percentage mortality in the current study was found in *C. gariepinus* fed the control group. In agreement with this result, Wang *et al.* [14] reported that dietary administration of *M. oleifera* leaf enhanced the post-challenge survival of common carp, *Cyprinus carpio* against *A. hydrophila* infection. *M. oleifera* plant has been reported to contain bioactive compounds such as nitrile, glycosides and thiocarbamate glycosides [21, 22]. The role of the bioactive compounds in the extract is to boost the non-specific defense system of the immune cells [13]. Therefore, it can be inferred that the addition of Moringa plant extracts in Moringa leaf supplemented diets (MLSC10, MLSC15, MLSC20 and MLSC25) reduced the effect of stress and improve growth and survival of *C. gariepinus* in the present study.

The present study showed that dietary supplementation with aqueous extract of Moringa leaves resulted in increased banding profile of the HSP 70 genes on the gel in *C. gariepinus* compared with the control, suggesting that the inclusion of Moringa increased the accumulation of the HSPs in fish after *A. hydrophila* and hyperthermia –induced stress. In another study on HSP expression after *E. ictaluri* infection, three HSP genes (HSP90, HSP70 and HSP60) were up-regulated with the significant up-regulation being detectable as early as 3 h after challenge in channel catfish [11]. The highly conserved heat shock proteins (HSPs) are expressed and function as molecular chaperones which facilitate the synthesis and folding of proteins [8]. This is in agreement with the more abundant expression and up-regulation of HSP70 in *C. gariepinus* used in the current study which corresponds with the supplementation of aqueous extracts of Moringa leaves especially in treatments MLSC10, MLSC15 and MLSC20. The elevated HSP 70 proteins expression has been associated with diseases typified by protein misfolding which includes degradation, disaggregation of stress-induced misfolding [8].

**CONCLUSIONS**

The present study confirmed the efficacy of *M. oleifera* leaf extract as a potent dietary supplement during thermal stressful periods in the aquaculture production of *C. gariepinus*. At the molecular *M. oleifera* leaf as dietary supplements helped to up-regulate the expression of the stress gene, Heat Shock Protein (HSP 70) in *C. gariepinus*. Based on the result of this study, a dose of 1.00g/kg dietary Moringa leaf supplementation per kg diet with a corresponding content of 0.21 mg /g and 0.14 mg /g Quercetin (Q) and Kaempferol (K), respectively was sufficient to prevent reduction in mortality and up-regulate the expression of HSP genes in *C. gariepinus*. Improvement of survival and health of *O. niloticus* and *C. gariepinus* fed the aqueous extract of *M. oleifera* leaves may be explained by the radical scavenging properties and metal ion chelation ability of the antioxidative phytochemicals such as the flavonoids mainly Quercetin (Q) and Kaempferol (K) present in the aqueous extract of the plant.
REFERENCES


