Organosomatic indices and tissues bacteriological changes of *Clarias gariepinus* on feeds supplemented with neem (*Azadirachta indica*) leaves and turmeric (*Curcuma longa*) rhizome extracts

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Abstract

The study evaluated the effect of dietary doses of neem leaves and turmeric rhizome extracts on tissue bacteriology and organosomatic indices of *Clarias gariepinus* juveniles. Nine experimental diets containing control (0%), NL 2 (1%), NL 3 (2%), NL 4 (3%), TR 5 (1%), TR 6 (2%), TR 7 (3%), CHRL 8 (15mg/kg diet) and CHRL 9 (30mg/kg diet) were formulated and replicated twice at 40% crude protein for 84 days. Microbial analyses of water, fish tissue and organ index of *C. gariepinus* were examined after 28, 56 and 84 days of feeding. Data were analyzed using ANOVA at p = 0.05. The results revealed that the fish fed treated experimental diets had decreased values of enterobacteriacea and total viable counts of bacterial loads from the water and fish tissues than the control at 28, 56 and 84 days. Turmeric rhizome extracts performed better when compared to the neem leaves and control. Also, organ index showed that liver, kidney and heart were not significantly increased in all the treated groups and the control. Neem leaves and turmeric rhizome extracts could be used in organic aquaculture to stimulate immunity, reduce and prevent bacterial infections in fish farming.

**Keywords:** Turmeric rhizome, Neem leaves, *Clarias gariepinus*, Microbial loads, Immunity, Chloramphenicol

Introduction

Fish are considered one of the important food sources for human being because their flesh contains a high percentage of protein, calcium, phosphorus, and iodine that are vital to our health (FAO, 2014). Fish account for almost 17 percent of the global production intake of animal protein (FAO, 2014). Fish diseases are major constraint to aquaculture production. Fish disease is a rarely simple association between pathogen, a host and environmental problems such as poor water quality, overcrowding or other stressors often contribute to the disease outbreaks (Mehana *et al*., 2015). The culture environment tends to produce poor physiological conditions for fish and increased susceptibility to infection. However, the use of chemicals and drugs in the aquaculture systems caused residual problems and poor quality in fish and its products. Food safety has become a major issue of concern in the developed countries (Sakai, 1999 and Chinabut and Puttinawarat, 2005). Therefore, looking for less harmful approaches and more environmentally – friendly treatments become of premium importance.

Natural medicinal products origin from fungi and herbs have been used as feed additives for farm animals in developed countries and showed many bioactive such as anti – fungi, antimicrobial, antioxidant, antiviral, immune enhancement and stress reduction (Wang *et al*., 1998; Odugbemi,
A natural medicinal product is a substance produced by a living organism which is found in nature and which usually has a biological or pharmacological activity that can be used in drug discovery and drug design. Natural medicinal products are important in the treatment of life threatening conditions (Gomathinayagam et al., 2014). Some medicinal plants/herbs had been evaluated experimentally in fish by various researchers, some with the use of specific part of the plant using various concentration/inclusion rate; Allium cepa (Bello et al., 2012), Tetraselmis chuii (Cerezuela et al., 2012), Viscum album (Park and Choi, 2012), Ficus benghalensis (Verma et al., 2012), Andrographis paniculata (Prasad and Mukthira, 2011), Euglena viridis (Das et al., 2009) and Allium sativum (Nya and Austin, 2009), but there is little or no information on utilization and mechanism of actions of A. indica and C. longa in fish nutrition. The present study aimed to evaluate the efficiency of using and as a potential antimicrobial agents in the organic aquaculture.

**Materials and methods**

**Plant collection and identification**
Azadirachta indica and C. longa were obtained in Igodan, Okitipupa, Ondo state and was identified by Dr. D. O. Aworinde in the Department of Biological Sciences, Ondo State University of Science and Technology, Okitipupa.

**Preparation and extraction of plant materials**

**Turmeric extraction**
The turmeric rhizome was washed with clean distilled water, scraped and allow to air dry at ambient temperature (25°C) for one hour. Three hundred grams (300g) of the air dried turmeric rhizome was blended and mixed with 1800 mL methanol (95%) and was stirred every 3 hours to obtain homogeneous extracts in the laboratory of Fisheries and Aquaculture, Ondo State University of Science and Technology, Okitipupa for 24 hours. The pulp obtained was filtered using a sterile muslin cloth after which the extracts was obtained, air-dried and stored (4°C) until required.

**Neem leaves extraction**
The extraction was done as described by Ajaiyeoba and Fadare (2006). The leaves were air dried and finely chopped. The air-dried neem leaves were ground with a hammer mill and 300g of fine powered of neem leaves were soaked in 1800 mL of ethanol (95%) for 48 hours. Neem leaves were properly mixed with ethanol filtered using a sterile muslin cloth after which the extract was obtained, air-dried and stored at (25°C) until required.

**Experimental system**
The experiment was carried out in eighteen plastic experimental tanks (50×34×27cm) for twelve (12) weeks in the Fisheries Laboratory of Ondo State University of Science and Technology, Okitipupa. The water level in each tank was maintained at volume of 35 litres throughout the experimental period. Water in each tank was replaced every three (3) days throughout the period of experiment to maintain relatively uniform physiochemical parameters and also to prevent fouling that may result from food residues. The source of water was from Ondo State University of Science and Technology, Okitipupa water station and each experimental tank was well aerated using air stone and aerator pumps (Lawson, 1995). The water temperature of the experimental tanks was measured by mercury –in-glass thermometer. While the pH value was measured using pH meter (Jenway 2013 pH meter, 0.01 accuracy) after standardizing the meter.
Experimental procedure and feeding trials
Each treatment has two replicates, 20 fish per replicate with mean initial body weight of 6.33±0.01g uniform-sized fish was selected from 400 juveniles. Weighed and distributed in experimental tanks. The fish was acclimated for eight days in experimental tanks before the experiment. The experiment last for 12 weeks during which the fish was fed at 3% body weight daily. The diet per day was divided into two; half was given in the morning by 8.00-9.00am and other half in the evening by 5.00pm. Measurement of the weight changes was performed monthly on fish organ (heart, liver and kidney) as well as determination of microbial loads in fish tissues and water.

Preparation of experimental diets
Feed ingredients such as fish meal, soybean, yellow maize, wheat bran, starch, vegetable oil, vitamin premix and bone meal were added and feed ingredients was mixed thoroughly in a mixer. Each diet mixture treated separately was extruded through a 1/4mm die mincer of Hobart A-200T pelleting machine to form a noodle like strand which was mechanically broken into suitable sizes for the C. gariepinus juveniles. The pelleted diets were sun dried, packed in labelled polythene bags and stored in a cool dry place to prevent mycotoxin formation. The mean proximate composition of the experimental diets were 40.1% crude protein, 7.1% moisture, 6.5% ether extract, 11.6%, 34.7% nitrogen free extract. Nine experimental diets were prepared by incorporating turmeric rhizome, neem leaves extracts and chloramphenicol at the following inclusion levels; 0 (control), 1%, 2%, 3% and 15mg/kg, 30mg/kg of the diet, respectively.

Media preparation
All media used were prepared according to manufacturer’s instruction. All these media are allowed to cool after sterilization to about 45°C before pouring into petri dishes.

Microbiological analysis
Water samples from the experimental bowls were collected monthly in sterile glass bottles. Distilled water was used for serial dilution; 1mL of water sample was added to 9mL sterile distilled water to 10^1 and then serially diluted to 10^4. Each diluent (1mL) was poured in two Petri dishes; one received nutrient agar for total viable counts using the pure plate count method according to the standard methods for the examination of water and wastewater (APHA, 1985), the second Petri dish received MacConky agar for enterobacteriacea counts according to Hitchins et al., (1995). Petri dishes were gently tapped on the sides for a few times, petri dish for enterobacteriacea counts and that of the dishes of total viable counts was incubated at 37°C for 24h.

Fish samples (skin, liver, gill and intestine) were collected monthly during the experimental period for bacteriological examination with through asepsis (medical examinations or procedures that prevent contamination or infection of microbes). 1g of fish sample was macerated in 9mL sterile peptone water in the mortar. 1mL of the suspension was diluted by peptone water to 10^4. Each diluent (1mL) was poured in two Petri dishes; one received nutrient agar and the other received MacConky agar (APHA, 1985). The incubation period was 24h at 37°C. After incubation of water and fish sample dishes the colonies were counted using colony counter. Total viable counts and enterobacteriacea counts were determined, the result were expressed in log_{10} CFU/mL and log_{10} CFU/g for water and fish, respectively.

Organ index
Three fish from each experimental treatment were killed by rapid cervical chopping and weighed. The liver, kidney...
and intestine were removed and weighed and the average was calculated. Moreover, organosomatic indices were calculated according to Fox et al. (1997). Organ –somatic index = [organ weight (g)/body weight (g)] X 100.

**Statistical analysis**

Microbial loads of water and fish tissues and organosomatic indices resulting from the experiment were subjected to one–way analysis of variance (ANOVA) Using SPSS (Statistical Package for Social Sciences 2006 Version 15.0). Duncan’s multiple range test was used to compare differences among individual means.

**Results**

**Microbial loads of fish tissues (skin, gill, liver and intestine)**

The highest enterobacteriacea and total viable counts were recorded in the control. The value decreased as the inclusion level increased and as the months increased in the treated groups (see Table 2A and 2B). The turmeric treated groups performed better when compared to neem and chloramphenicol treated groups.

### Table 2A: The result of the enterobacteriacea and total viable counts (log_{10}CFU/g) of C. gariepinus treated with turmeric rhizome and neem leaves extracts

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fish sites</th>
<th>Enterobacteriacea counts</th>
<th>Total viable counts</th>
<th>Enterobacteriacea counts</th>
<th>Total viable counts</th>
<th>Enterobacteriacea counts</th>
<th>Total viable counts</th>
<th>Enterobacteriacea counts</th>
<th>Total viable counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Intestine</td>
<td>3.75±0.01</td>
<td>3.80±0.00</td>
<td>3.65±0.00</td>
<td>3.72±0.01</td>
<td>3.58±0.03</td>
<td>3.68±0.04</td>
<td>3.72±0.01</td>
<td>3.68±0.04</td>
</tr>
<tr>
<td>Skin</td>
<td>3.96±0.04</td>
<td>4.02±0.02</td>
<td>3.85±0.01</td>
<td>3.95±0.00</td>
<td>3.71±0.00</td>
<td>3.89±0.02</td>
<td>3.71±0.00</td>
<td>3.89±0.02</td>
<td>3.71±0.00</td>
</tr>
<tr>
<td>Gill</td>
<td>3.88±0.02</td>
<td>3.92±0.03</td>
<td>3.79±0.00</td>
<td>3.83±0.02</td>
<td>3.65±0.00</td>
<td>3.77±0.03</td>
<td>3.65±0.00</td>
<td>3.77±0.03</td>
<td>3.65±0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>3.81±0.01</td>
<td>3.86±0.04</td>
<td>3.79±0.02</td>
<td>3.85±0.03</td>
<td>3.65±0.01</td>
<td>3.75±0.04</td>
<td>3.65±0.01</td>
<td>3.75±0.04</td>
<td>3.65±0.01</td>
</tr>
</tbody>
</table>

### Table 2B: Enterobacteriacea and total viable counts (log_{10}CFU/g) of C. gariepinus treated with chloramphenicol

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fish sites</th>
<th>Enterobacteriacea counts</th>
<th>Total viable counts</th>
<th>Enterobacteriacea counts</th>
<th>Total viable counts</th>
<th>Enterobacteriacea counts</th>
<th>Total viable counts</th>
<th>Enterobacteriacea counts</th>
<th>Total viable counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Intestine</td>
<td>3.75±0.01</td>
<td>3.80±0.00</td>
<td>3.65±0.00</td>
<td>3.72±0.01</td>
<td>3.58±0.03</td>
<td>3.68±0.04</td>
<td>3.72±0.01</td>
<td>3.68±0.04</td>
</tr>
<tr>
<td>Skin</td>
<td>3.96±0.04</td>
<td>4.02±0.02</td>
<td>3.85±0.01</td>
<td>3.95±0.00</td>
<td>3.71±0.00</td>
<td>3.89±0.02</td>
<td>3.71±0.00</td>
<td>3.89±0.02</td>
<td>3.71±0.00</td>
</tr>
<tr>
<td>Gill</td>
<td>3.88±0.02</td>
<td>3.92±0.03</td>
<td>3.79±0.00</td>
<td>3.83±0.02</td>
<td>3.65±0.00</td>
<td>3.77±0.03</td>
<td>3.65±0.00</td>
<td>3.77±0.03</td>
<td>3.65±0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>3.81±0.01</td>
<td>3.86±0.04</td>
<td>3.79±0.02</td>
<td>3.85±0.03</td>
<td>3.65±0.01</td>
<td>3.75±0.04</td>
<td>3.65±0.01</td>
<td>3.75±0.04</td>
<td>3.65±0.01</td>
</tr>
</tbody>
</table>

The mean values in each row with similar superscripts are not significantly different (p > 0.05), TR= Turmeric (rhizome) treatment, NL = Neem Leaves.
Olusola, Olawoye and Olaifa

**Microbial analysis of water**

Enterobacteriacea counts and total viable counts were higher in control than the treated groups. Enterobacteriacea and total viable counts were significant different (P>0.05) in all the treated groups of the experiment (Table 3).

<table>
<thead>
<tr>
<th></th>
<th>Enterobacteriacea counts</th>
<th>Total viable counts</th>
<th>Enterobacteriacea counts</th>
<th>Total viable counts</th>
<th>Enterobacteriacea counts</th>
<th>Total viable counts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Week 4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.15±0.09</td>
<td>2.30±0.05</td>
<td>2.29±0.06</td>
<td>2.43±0.15</td>
<td>2.17±0.02</td>
<td>2.31±0.00</td>
</tr>
<tr>
<td>TR2</td>
<td>1.93±0.05</td>
<td>2.32±0.14</td>
<td>2.39±0.05</td>
<td>2.56±0.10</td>
<td>2.48±0.01</td>
<td>2.69±0.01</td>
</tr>
<tr>
<td>TR3</td>
<td>2.15±0.24</td>
<td>2.48±0.02</td>
<td>2.16±0.38</td>
<td>2.21±0.22</td>
<td>1.74±0.02</td>
<td>2.00±0.02</td>
</tr>
<tr>
<td>TR4</td>
<td>2.05±0.14</td>
<td>2.36±0.15</td>
<td>2.24±0.20</td>
<td>2.28±0.01</td>
<td>1.78±0.3</td>
<td>1.82±0.03</td>
</tr>
<tr>
<td>NL5</td>
<td>2.25±0.10</td>
<td>2.59±0.01</td>
<td>2.34±0.00</td>
<td>2.46±0.06</td>
<td>2.08±0.01</td>
<td>2.21±0.02</td>
</tr>
<tr>
<td>NL6</td>
<td>2.26±0.00</td>
<td>2.35±0.14</td>
<td>2.11±0.14</td>
<td>2.45±0.03</td>
<td>2.26±0.01</td>
<td>2.57±0.02</td>
</tr>
<tr>
<td>NL7</td>
<td>2.32±0.08</td>
<td>2.47±0.22</td>
<td>2.20±0.00</td>
<td>2.94±0.08</td>
<td>2.39±0.02</td>
<td>2.52±0.03</td>
</tr>
<tr>
<td>CHL8</td>
<td>2.44±0.04</td>
<td>2.56±0.03</td>
<td>2.41±0.07</td>
<td>2.65±0.00</td>
<td>1.78±0.01</td>
<td>1.88±0.04</td>
</tr>
<tr>
<td>CHL9</td>
<td>2.22±0.31</td>
<td>3.23±0.04</td>
<td>2.33±0.13</td>
<td>2.48±0.15</td>
<td>1.85±0.02</td>
<td>1.93±0.03</td>
</tr>
</tbody>
</table>

The mean values in each row with similar superscripts are not significantly different (p > 0.05), TR= Turmeric (rhizome) treatment, NL=Neem leaf treatment, CHR=chloramphenicol treatment

**Organ index of C. gariepinus fed experimental diets for 84 days**

The result reveals that the treated groups had better organ index when compared to the control. The value obtained in liver, kidney and heart were closely related and there were no significantly different (p>0.05) among the treatments (see table 4).

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table 4: Organ index of C. gariepinus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>0.40± 0.01</td>
<td>0.15± 0.02</td>
<td>0.12± 0.01</td>
</tr>
<tr>
<td>Control</td>
<td>0.35± 0.02</td>
<td>0.15± 0.01</td>
<td>0.10± 0.00</td>
</tr>
<tr>
<td>TR2</td>
<td>0.30± 0.01</td>
<td>0.10± 0.00</td>
<td>0.10± 0.01</td>
</tr>
<tr>
<td>TR3</td>
<td>0.30± 0.00</td>
<td>0.13± 0.02</td>
<td>0.10± 0.01</td>
</tr>
<tr>
<td>TR4</td>
<td>0.30± 0.01</td>
<td>0.11± 0.00</td>
<td>0.10± 0.00</td>
</tr>
<tr>
<td>NL5</td>
<td>0.25± 0.00</td>
<td>0.10± 0.01</td>
<td>0.10± 0.01</td>
</tr>
<tr>
<td>NL6</td>
<td>0.20± 0.02</td>
<td>0.11± 0.00</td>
<td>0.11± 0.00</td>
</tr>
<tr>
<td>NL7</td>
<td>0.20± 0.02</td>
<td>0.10± 0.01</td>
<td>0.10± 0.01</td>
</tr>
<tr>
<td>CHR8</td>
<td>0.25± 0.01</td>
<td>0.10± 0.00</td>
<td>0.05± 0.01</td>
</tr>
<tr>
<td>CHR9</td>
<td>0.20± 0.00</td>
<td>0.10± 0.01</td>
<td>0.10± 0.00</td>
</tr>
</tbody>
</table>

The mean values in each column with similar superscripts are not significantly different (p > 0.05), TR= Turmeric rhizome, NL= Neem leaves, CHR= Chloramphenicol treatment

**Discussion**

The result obtained in this study showed the presence of saponins, flavonoids, glucosinlates, amino acids and polysterols in neem leaves and turmeric rhizome extracts but tannin and phenol were not detected neem leaves and turmeric rhizome. Environmental factors and the methods of preparation samples may influence the concentration of tannin and phenol. The concentrations of these metabolites in neem leaves and turmeric rhizome extracts were available in abundant and moderate quantities. This
support the report of Njoku et al. (2009) that plants contain chemical compounds such as saponins, tannins, flavonoids, glucosinolates, phenol, amino acids and polysterols known as metabolites which are biologically active. The phytochemicals such as tannins, saponins and glucosinolates present in medicinal plants may have antimicrobial activity (Priya et al., 2012).

In fish health managements, the most useful index of success is prevention of disease and systematic physicochemical analysis of the water and monitoring of the microorganism in aquaculture system (Krishna et al., 2012). This study revealed that the microbial loads in intestine, skin, gill and liver of C. gariepinus varies with the skin and gill having highest value of total viable counts and enterobacteriaceae counts. The highest enterobacteriaceae and total viable counts were recorded in control. The value decreased as the inclusion level increased and as the month increased this support the report of Shalaby et al. (2006) and Bello et al. (2012). Investigation demonstrated that fresh water fishes were affected by infection with different types of bacteria (Khatun et al., 2011). Bacteria are greater on skin than any part of the body as these parts are constantly exposed to challenges. However, gut contains more bacteria load than surrounding water (Khatun et al., 2011). The antimicrobial effect of turmeric rhizome and neem leaves extracts in the diets lead to reduction in the microbial loads of fish tissues (skin, gill, liver and intestine) and inhibited the growth of microorganism that result in infection of fish. This might be attributed to the presence of antimicrobial properties in turmeric rhizome and neem leaves extracts (saponins, tannins and flavonoids).

Microbial analysis of water showed that the enterobacteriaceae populations in water were higher in control than the treated groups containing turmeric rhizome, neem leaves and chloramphenicol at 4, 8 and 12 weeks. Observations revealed that the bacterial loads in the water of the experimental tanks were affected by the presence of C. longa and A. indica in the diets than control. This observation was supported by the findings of Shalaby et al. (2006) and Bello et al. (2012). The organosomatic indices are indicator of health which could be used to predict the health status of fish (Fox et al., 1997). The results of organ indices showed that the liver, heart and kidney were not significantly (p > 0.05) increased in all the treated groups. Although, the result of the liver showed that there were significant differences (p < 0.05) among the treatments while the kidney and heart were not significantly different (p > 0.05). The finding agreed with the report of Abd-El-Rhman (2009) and Bello et al. (2012). The findings showed no traces of oedema and high variation of the intestinal organs, the inclusion of turmeric rhizome and neem leaves extracts in the diet of C. gariepinus could therefore be considered safe and non-toxic for consumption.

Conclusion and recommendation
Application of turmeric rhizome and neem leaves extracts in feed reduced the microbial populations in water and fish tissues. The relative abundance and availability of turmeric rhizome and neem leaves makes them cheap, natural, nutritional and antimicrobial natural product to be explored in aquaculture. Further research should be carried out on interaction /synergistic effects of turmeric rhizome and neem leaves extracts on C. gariepinus and other species of fish.

Acknowledgement
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