Sokoine University of Agriculture

MSc. Dissertation

Effects of Neem (*Azadirachta Indica*) Leaf Powder on Intestinal Histomorphology and Growth Performance in Nile Tilapia (*Oreochromis niloticus*) Fingerlings

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May 2024

EFFECTS OF NEEM (*AZADIRACHTA INDICA*) LEAF POWDER ON INTESTINAL HISTOMORPHOLOGY AND GROWTH PERFORMANCE IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*) FINGERLINGS

A Dissertation Submitted In Partial Fulfillment of the Requirement for the Degree of Masters of Science in Health of Aquatic Animal Resources at Sokoine University of Agriculture. Morogoro, Tanzania.

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EXTENDED ABSTRACT

The application of leaves from neem tree (*Azadirachta indica*) as dietary feed additive in Nile tilapia (*Oreochromis niloticus*) fish is gaining attention worldwide due to its effectiveness and minimal side effects on the aquatic environment. As a phytomedicine has been included in juvenile Nile tilapia diets at inclusion levels of 2–8 g/kg feed to control reproduction, by exerting degenerative histological changes on gonadal tissues of Nile tilapia fish. However, a slow growth rate and lowered feed utilization were reported in juvenile Nile tilapia post-exposure to neem compounded feeds. Therefore, this study aimed to evaluate the effects of neem leaf powder (NLP) on intestinal histomorphology in tilapia fish and its association with growth performance. A total of 180 Nile tilapia fingerlings were randomly distributed in four groups ($n = 30$ for growth performance, $n = 15$ for intestinal histomorphology) and kept in a clear-water recirculation aquaculture system (RAS). A total of 45 fingerlings used in control groups, while 135 used in treated groups. The fish received NLP at concentrations of 0 g, 1 g, 4 g, and 8 g for each kilogram of Nile tilapia commercial feed and the experiment was conducted for 90 days. Results showed that the inclusion of NLP at 4 g/Kg feed significantly ($P < 0.05$) enhanced weight gain (WG), percent weight gain (PWG), daily weight gain (DWG), and specific growth rate (SGR) in comparison to treated groups given NLP 1 g and 8 g/kg feed and the control group. A higher survival rate ($P < 0.05$) was recorded in the group fed NLP at 8 g/Kg feed. Values for intestinal histomorphometry (villous height) in the middle/spiral intestine in the group fed 8 g/Kg feed were significantly lower ($p < 0.05$) than in the control group. Moreover, compared to the control group, the treated group on early exposure to NLP (Days 0–3) had increased TNF-α and single-stranded DNA (ssDNA) expression. The TNF-α was associated with significant ($p < 0.05$) increased expression of TNF-α in all portions of the intestines and pancreatic regions compared. This was reflected

by the inflammatory effect in the proximal, middle, and distal intestines. But on long-term exposure (Day 30–90), all treated groups and all three intestinal portions experienced epithelial recovery associated with decreased expression of TNF-α and ssDNA. In conclusion, there was an adaptation in long-term exposure to the used NLP doses on Nile tilapia fingerlings, and the inclusion from one up to 4 g of NLP per Kg feed has no detrimental effect on intestinal morphology and promotes growth and feed utilization.

Keywords: Nile tilapia; Neem plant; Intestine; Histomorphology; TNF-α; ssDNA; Growth performance; Histomorphometry

IKISIRI KUU

Matumizi ya majani ya mti wa mwarobaini (*Azadirachta indica*) kama nyongeza ya lishe katika samaki wa Sato (*Oreochromis niloticus*) yanapata umaarufu ulimwenguni kutokana na ufanisi wake na athari ndogo kwa mazingira ya maji. Kama dawa ya mimea imewekwa katika lishe ya samaki wadogo wa Sato kwa viwango vya 2-8 g/kg ya chakula ili kudhibiti uzazi, kwa kusababisha mabadiliko ya kihistolojia kwenye tishu za uzazi za samaki wa Sato. Hata hivyo, kasi ndogo ya ukuaji na utumiaji wa chakula uliopungua uliripotiwa kwa samaki wadogo wa Sato baada ya kutumia lishe iliyochanganywa na mwarobaini. Kwa hivyo, utafiti huu ulilenga kutathmini athari za unga wa majani ya mwarobaini (NLP) kwenye histomofoloji ya utumbo wa samaki wa tilapia na uhusiano wake na utendaji wa ukuaji. Jumla ya vifaranga vya Sato 180 viligawiwa kwa njia isiyo ya kawaida katika vikundi vinne (n = 30 kwa utendaji wa ukuaji, n = 15 kwa histomoroloji ya utumbo) na kuwekwa katika mfumo wa ufugaji wa samaki wa mzunguko wa maji safi (RAS) kwa siku tisini (90). Vifaranga 45 wakigawanywa katka kundi la kudhibitiwa na 135 wakigawanywa kwenye vikundi vilivyolishwa NLP. Samaki walipokea NLP kwa viwango vya 0 g, 1 g, 4 g, na 8 g kwa kila kilogramu ya chakula cha biashara cha Sato na majaribio yalifanyika kwa siku 90. Matokeo yalionyesha kuwa uingizaji wa NLP kwa 4 g/Kg ya chakula uliongeza sana ($P < 0.05$) ongezeko la uzito (WG), asilimia ya ongezeko la uzito (PWG), ongezeko la uzito kila siku (DWG), na kiwango maalum cha ukuaji (SGR) ikilinganishwa na vikundi vilivyolishwa kwa NLP 1 g na 8 g/kg ya chakula na kundi la kudhibitiwa. Kiwango kikubwa cha kuishi ($P < 0.05$) kilirekodiwa kwenye kundi lililolishwa NLP kwa 8 g/Kg cha chakula. Thamani za histomofometri ya utumbo (urefu wa vili) katika utumbo wa kati/spiral katika kundi lililolishwa 8 g/Kg cha chakula zilikuwa chini sana ($p < 0.05$) ikilinganishwa na kundi la kudhibitiwa. Zaidi ya hayo, ikilinganishwa na kikundi cha udhibiti, kikundi kilicholishwa kwa NLP (Siku 0-3) kilionyesha kuongezeka TNF-α na ssDNA katika sehemu za utumbo. TNF-α ilihusishwa na ongezeko kubwa ($p < 0.05$) katika sehemu zote za utumbo na maeneo ya tezi ya kongosho ikilinganishwa. Hii ilionyeshwa

na athari ya uchochezi katika sehemu za utumbo wa juu, utumbo wa kati, na wa chini. Lakini kwa mfiduo wa muda mrefu (Siku 30-90), vikundi vyote vilivyolishwa NLP na sehemu zote tatu za utumbo vilipata urejesho wa epithelial uliohusishwa na kupungua kwa TNF-α na ssDNA. Kwa hitimisho, kulikuwa na mabadiliko kwa mfiduo wa muda mrefu kwa viwango vilivyotumika vya NLP kwa vifaranga wa samaki wa Sato, na kuongezwa kutoka gramu moja hadi 4 za NLP kwa Kg ya chakula hakuna athari mbaya katika utumbo na inakuza ukuaji na matumizi ya chakula.

Maneno muhimu: Histomofometri; Histomofoloji; Mwarobaini; Sato; ssDNA; TNF-α; Utendaji wa Ukuaji; Utumbo

DECLARATION

I **Wilbert Gaudence Mitao,** do hereby declare to the senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution

_____ _____

Wilbert Gaudence Mitao Date

(MSc. Candidate)

The above declaration is confirmed by:

_____ _____

Prof. Claudius. Luziga Date

(Supervisor)

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Prof. Robinson. Mdegela Date

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LIST OF MANUSCRIPT

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DEDICATION

This dissertation is dedicated to my family and all other relatives who made my career successful by investing their time and resources in me.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µm	Micrometer
ANOVA	Analysis of Variance
cm	Centimeter
DPX	Distyrene, Plasticizer (Tricresyl Phosphate) and Xylene
g/Kg	gram per Kilogram
H&E	Hematoxylin and Eosin
mg/L	Milligram per Liter
NLP	Neem Leaf Powder
PBS	Phosphate-Buffered Saline
RAS	Recirculation Aquaculture System
SPSS	Statistical Package of Service Solution
SsDNA	Single-stranded DNA
SUA	Sokoine University of Agriculture
TNF-α	Tumor Necrotic Factor-alpha

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background Information

Tilapia fish have become the most extensively cultivated fish globally, experiencing a significant increase in production over recent decades. This surge can be attributed to its suitability for aquaculture conditions, high market demand, and stable pricing (Ng and Romano, 2013). Predominantly farmed in tropical and subtropical regions, Tilapia constitutes the largest group of cultured finfish (El-Sayed *et al*., 1999). In Tanzania, farmers primarily rear mixed-sex Tilapia species in earthen ponds, often obtaining their seed stock from wild sources or neighboring farms (Mndeme *et al*., 2020).

The popularity of Tilapia among farmers stems from its ability to thrive in crowded conditions, its marketability, ease of captive spawning, and high disease resistance (Shiau, 2002). Moreover, Tilapia exhibits adaptability to a wide range of feeds, offering cost-effective options for intensive farming systems (Audu *et al*., 2010). This adaptability is attributed to its long intestines, which facilitate the digestion of plant-based feeds (Audu *et al*., 2010). Additionally, Nile Tilapia possesses favorable intestinal histological features, including an extensive absorptive surface area and increased mucosal folds, particularly in the proximal portion of the intestine, which is crucial for digestion and absorption (Gargiulo *et al*., 1998; Plaul *et al*., 2016).

However, a significant challenge associated with Nile Tilapia farming is premature spawning, leading to overpopulation and diversion of resources towards reproduction rather than somatic growth, resulting in stunted growth and lower market weights (Jegede and Fagbenro, 2008; Kareem *et al*., 2016). To address this issue, neem leaves have been

introduced as a natural antifertility agent, with inclusion levels of 2 to 8 g/kg feed effectively controlling overpopulation habits (Obaroh and Achionye-Nzeh, 2011; Kapinga *et al*., 2019).

Neem leaves have gained global attention in aquaculture due to their efficacy and minimal environmental impact, surpassing other herbal alternatives because of their potent bioactive compounds (Mousa *et al*., 2008). They have been utilized to enhance fish immunity against various pathogens, such as *Vibrio harveyi* and *Aphanomyces invadans*, as well as exhibiting potent antibacterial activity against *Vibrio parahaemolyticus* (Banerjee *et al*., 2013; Talpur and Ikhwanuddin, 2013). Moreover, neem leaves have been proven effective in terrestrial animals for treating microbial and parasitic infections (Tibebu *et al*., 2017).

Neem leaves are known to be eco-friendly, with no adverse effects on water quality or phytoplankton communities when applied in aquatic environments (Thanh Binh *et al*., 2016). Compared to synthetic antiparasitic agents, neem leaves have fewer toxic side effects (Mousa *et al*., 2008). Research has shown that incorporating neem leaves, along with other plant by-products like guava and bitter leaf extracts, into Nile Tilapia diets can significantly improve growth performance (Abarike *et al*., 2022). Despite the recognized benefits of neem leaves in aquaculture, little is known about their effects on the gastrointestinal tract (GIT) of Tilapia fish. Therefore, this study aims to investigate the effects of neem leaves on the intestinal histomorphology of Nile Tilapia fingerlings and its correlation with growth performance.

1.2 Literature Review

1.2.1 Neem description and morphology

Neem (*Azadirachta indica*), known as Nimtree in English and Mwarobaini in Tanzania, belongs to the Meliaceae family (Kashif and

Ullah, 2013). Various parts of the neem tree, including leaves, seed cake, and seed oil cake, are utilized (Heuzé *et al*., 2012). Originating from Burma in India, the neem tree finds its historical roots (Kapinga *et al*., 2019). In Africa, its initial application was as an insecticide within the aquaculture industry (Khoa *et al*., 2019). The tree maintains its green foliage year-round and typically reaches heights between 15 to 30 meters, featuring a broad circular crown spanning 10 to 20 meters in diameter (Heuzé *et al*., 2012). Its leaves, concentrated at branch tips, are characterized by indentations, sickle shapes, and a dark green hue upon maturity (Heuzé *et al*., 2012).

1.2.2 Neem utilization

The Neem tree has garnered global attention in both human and animal applications due to its abundance of phytochemicals with potent antimicrobial properties (Rebecca, 2014). Variations in the concentration of these phytochemical active compounds across different parts of the neem tree necessitate varying doses depending on the specific plant part utilized (Winkaler *et al*., 2007). Consequently, the biochemical attributes of these phytochemical compounds, potentially imbued with medicinal effects, have found utility in aquatic veterinary practices, often at low concentration rates as substitutes for antimicrobial agents (Heuzé *et al*., 2012).

Neem leaves are known for their antifungal, antiviral, and anti-inflammatory properties (Raut *et al*., 2014). Additionally, they stimulate the production of red blood cells (RBCs), white blood cells (WBCs), and antibodies, thereby bolstering immunity (Logambal and Dinakaran, 2000; Carneiro *et al*., 2008). However, incorporating neem seed cake at 10% levels in feed reduces feed intake in terrestrial animals, although studies have indicated that up to 15% does not adversely affect amylase and protease enzymes in fish (Heuzé *et al*., 2012). The unpalatability and toxicity of neem leaves have limited their use in terrestrial livestock feeding due to the presence of anti-nutritional

factors, which can lead to stunted growth and mortality in monogastric species (Heuzé *et al*., 2012). Similarly, feeding neem leaves to ruminant animals has been associated with growth retardation (Biswas *et al*., 2002). Furthermore, therapeutic applications of neem in humans have been linked to adverse health effects in children (Heuzé *et al*., 2012).

However, combining neem leaves with other feed ingredients has been shown to enhance fish survival rates and growth (Dinda *et al*., 2020; Patel *et al*., 2021). In rainbow trout, optimal supplementation with neem leaf at 7% has proven cost-effective in boosting fish growth (Abidin *et al*., 2022). Recently, neem leaves have gained global traction in tilapia farming, where they serve as an antifertility agent in Nile tilapia (*Oreochromis niloticus*), addressing issues of premature spawning, overpopulation, and stunted growth (Kapinga *et al*., 2019).

1.2.3 Neem leaves histologic effect in fish

Neem leaf extract application in Nile tilapia fish has been found to induce severe necrosis in ovaries at higher doses, as evidenced by (Obaroh and Achionye-Nzeh 2011). Additionally, (Winkaler *et al*. 2007) observed damaged gill and renal tissues in tilapia fish exposed to doses of 2.5 g/l of neem leaf extract. The potent spermicidal effect of neem leaf extract has been utilized to achieve controlled breeding of tilapia fish (Kapinga *et al*., 2019), although contrasting effects, such as an increased fecundity rate, have been reported in *Cyprinus carpio* fish (Dinda *et al*., 2020; Kaur *et al*., 2020). Despite these findings, limited information exists regarding the intestinal histological effects of neem leaf powder in Nile tilapia fish.

1.2.4 Tilapia fish description and growth performance

Efforts to enhance tilapia species involved crossbreeding Mozambique tilapia and gift tilapia, resulting in red tilapia. However, this breed faced rejection from most farmers due to its tendency for early maturation,

prolific breeding leading to overpopulation in ponds, and stunted growth (Uddin *et al*., 2021). Tilapia typically achieve an average weight of approximately 5 kg, with males exhibiting faster growth in size and weight, and both sexes having a maximum lifespan of up to 10 years (Abdulhadi *et al*., 2022). Their coloration ranges from grey-brownish to dark grey, and they possess gill rackers numbering from 14 to 28, which are characteristic traits of Nile tilapia (Mndeme *et al*., 2020). Research indicates that the growth rate of Nile tilapia can be influenced by variations in water physicochemical parameters, including dissolved oxygen, transparency, temperature, and pH, in the aquatic environment (Alhassan *et al*., 2018). Moreover, the application of crude extracts of neem leaf saponins in juvenile Nile tilapia has been linked to an enhanced rate of material transport across intestinal mucosal cells, potentially promoting the growth performance of Nile tilapia fish (Obaroh *et al*., 2014). Nonetheless, there is limited understanding regarding the impact of neem leaf powder on the growth performance of Nile tilapia fingerlings when incorporated into feeds.

1.2.5 Tilapia fish intestinal histology and immunohistochemistry

The morphology and function of fish digestive systems vary widely, influenced by factors such as taxonomy classification and adaptation to feeding behavior (Abdulhadi *et al*., 2022). For instance, the intestinal structure of tilapia fish includes tubular glands, epithelial mucosa, layers of connective tissue, and muscular layers (Gargiulo *et al*., 1998). The inner arrangement comprises the lamina propria, submucosa, muscular layers, and outer serosal layer (Abdulhadi *et al*., 2022). Muscular layers aid in peristalsis, while goblet cells in the proximal intestine contribute to mucus production (Gargiulo *et al*., 1998). Blood perfusion in the lamina propria supports intestinal epithelial functions (Gargiulo *et al*., 1998). Furthermore, differences exist between proximal and distal intestines, with the former possessing more folds and villi for increased surface area (Abdulhadi *et al*., 2022). The fish intestine plays a critical role in immunity, affecting growth performance and disease resistance (Sitjà-

Bobadilla *et al*., 2019). Maintaining intestinal integrity is vital for overall fish health, especially considering exposure to pollutants and dietary interventions (Plaul *et al*., 2016; Cain and Swan, 2010). Additionally, TNF-α is implicated in intestinal barrier regulation and inflammation modulation (Leppkes *et al*., 2014).

TNF-α levels reflect the fish's response to intestinal injury, correlating with inflammation severity (Plaul *et al*., 2016; Sitja-Bobadilla *et al*., 2016). Neem leaf extract, known to influence TNF-α expression, has shown both pro-inflammatory and anti-inflammatory effects in different studies (Dewanti, 2011; Ruslie *et al*., 2020). However, there is limited data on immunohistochemical analysis in tilapia fish intestines, particularly concerning neem leaf powder exposure. This study aims to address this gap by investigating the effects of neem leaf powder in Nile tilapia fingerling intestines.

1.3 Problem Statement and Justification

Neem leaves have become widely used in aquaculture, however, its effect of causing stunted growth and rare fish mortality may pause a failure to meet the country’s goal of producing 250 million fingerlings per year by 2026 (AFDP, 2020). For instance, when lower than 1 g/kg feed and higher than 8 g/kg feed doses of neem leaves were administered in juvenile Nile tilapia, slow growth and feed utilization was observed (Kapinga *et al*., 2018). Little is thus known about the effects of neem leaves on Nile Tilapia fish intestinal histomorphology, nutrient absorption and associated growth performance. Henceforth, this study aimed at investigating the effects of neem leaves on Tilapia GIT and thereby establish safer inclusion dosage levels of neem leaf powder to be used in Tilapia fish by farmers and hatchery units.

1.4 Objectives

1.4.1 Broad objective

To determine the effects of neem (*Azadirachta indica*) leaf powder on the intestinal histomorphology of Nile Tilapia (*Oreochromis niloticus*) fingerlings and its relation to growth performance.

1.4.2 Specific objective

i. To evaluate the effect of neem (*Azadirachta indica*) leaf powder on growth performance in Nile tilapia (*Oreochromis niloticus*) fingerlings.
ii. To establish the effect of various neem (Azadirachta indica) leaf powder concentrations in Nile tilapia (*Oreochromis niloticus*) fingerlings intestinal histomorphology.

1.5 Research Questions/Hypothesis

The inclusion of Neem (*Azadirachta indica*) leaf powder in fish diets significantly affects the intestinal histomorphology and growth performance of Nile tilapia (*Oreochromis niloticus*) fingerlings.

CHAPTER TWO

MANUSCRIPT I

2.0 Effect of Neem (*Azadirachta indica*) Leaf Powder Exposure on Intestinal Histomorphometry and Growth Performance in Nile Tilapia (*Oreochromis niloticus*) Fingerlings

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ABSTRACT

Leaves from the neem tree (*Azadirachta indica*) are widely used as dietary feed additives in Nile tilapia (*Oreochromis niloticus*) due to their minimal side effects on the aquatic environment. However, a slow growth rate and lowered feed utilization were reported in juvenile Nile

tilapia post-exposure to neem compounded feeds. Little is known about the effects of neem leaves on fish gastrointestinal structure and feed intake. Therefore, this study was carried out in Morogoro Municipal, in Tanzania in 2023 to evaluate the effects of neem leaf powder (NLP) on Nile tilapia fingerlings intestinal histomorphometry and study its association with growth performance. A total of 180 Nile tilapia fingerlings were randomly distributed in four groups (n = 45) and kept in a clear-water recirculation aquaculture system (RAS). The fish received NLP at concentrations of 0 g, 1 g, 4 g, and 8 g for each kilogram of Nile tilapia commercial feed and the experiment was conducted for 90 days. Results showed that the inclusion of NLP at 4 gKg^{-1} feed significantly ($P < 0.05$) enhanced weight gain (WG), percent weight gain (PWG), daily weight gain (DWG), and specific growth rate (SGR) in comparison to untreated control group 0 g and treated groups given NLP 1 g and 8 gKg^{-1} feed. A higher survival rate ($P < 0.05$) was observed in the treated group fed on NLP at 8 gKg^{-1} feed, but it was lower in groups given NLP at 1 gKg^{-1} and 4 gKg^{-1} feed. Values for intestinal histomorphometry (villous height) in the middle/spiral intestine in the group fed 8 gKg^{-1} feed were significantly lower ($p < 0.05$) than the group fed 4 gKg^{-1} feed and the control group. In conclusion, administration of NLP at a dose of 1 gKg^{-1} up to 4 gKg^{-1} feed has been shown to promote feed utilization and growth rate without affecting intestinal morphology.

Keywords: Growth rate; Intestinal morphometry; Neem tree; *Oreochromis niloticus*

2.1 Introduction

Nile tilapia (*Oreochromis niloticus*) is among the most popular aquaculture-farmed fish globally (Miao and Wang 2020). In Tanzania, more than 95% of farmers grow mixed-sex tilapia species in earthen ponds and obtain their tilapia seeds for stocking in ponds from the wild

or neighboring fish farmers (Mndeme et al. 2020). Tilapia fish can be raised by different farming systems, such as semi-intensive or extensive systems in cages and ponds (Gullian-Klanian and Arámburu-Adame 2013). Clear-water recirculation aquaculture system (RAS) is mostly preferred for commercial large-scale production, starting with juvenile fish at a stocking density of up to 300 fish per cubic meter (Bhujel 2013). RAS allows farmers in urban and peri-urban areas to raise fish intensively and hyper-intensively with minimal water volume usage due to reconditioning and reusing (Gullian-Klanian and Arámburu-Adame 2013). When raised in RAS, fish growth performance of 69 g weight gain is achieved in 70 days (Gullian-Klanian and Arámburu-Adame 2013).

The gastrointestinal tract (GIT) of tilapia fish is an important organ for the absorption of nutrients, growth, and welfare status of the fish (Okuthe and Bhomela 2021). In particular, intestinal tissue integrity is of vital importance in enhancing fish growth, health, and welfare (Cain and Swan 2010). Three distinctive regions of the Nile tilapia intestine include proximal, middle, and distal; in normal anatomy, intestinal villi height increases from the distal through the middle to the proximal intestine (Pirarat et al. 2011; Okuthe and Bhomela 2021). Nile tilapia, as an omnivorous fish species, consumes both floating and bottom feeds and can feed on small invertebrates, aquatic plants, benthic fauna, and phytoplankton feeds (Bhujel 2013; Teixeira 2023).

However, the major setback in Nile tilapia is precocious spawning; hence, this phenomenon leads to overpopulation, which causes the wastage of feed resources on reproduction, instead of somatic growth (Kapinga et al. 2019). As a result, fish show stunted growth and low market weights (Jegede and Fagbenro 2008; Kareem et al. 2016). To solve that problem, neem (*Azadirachta indica*) leaf powder (NLP) as a

phytomedicine was introduced in fish diets at inclusion levels of 2 to 8 gKg^{-1} feed as an antifertility agent to control reproduction, hence limiting overpopulation (Obaroh and Achionye-Nzeh 2011; Kapinga et al. 2019). The leaves contain biologically active compounds with minimal side effects on the aquatic environment (Mousa et al. 2008; Kapinga et al. 2019). Neem leaves are therefore widely used as a dietary feed additive, as phytomedicine is, and the tree is highly available in the tropical zone (Kapinga et al. 2019; Adjabi and Adjina 2022).

Moreover, a slow growth rate and lowered feed utilization were reported in juvenile Nile tilapia post-exposure to neem compounded feeds (Kapinga et al. 2018). Little is known about its effect on the structure of gastrointestinal tissues and growth performance in Nile tilapia fingerlings. Fish intestinal morphology is strongly related to growth performance (Kim 2017; Teixeira 2023). The hypothesis is that there is a strong association between fish intestine morphology, feed utilization, and growth performance. Therefore, this study aimed to evaluate the effects of neem leaf powder (NLP) on Nile tilapia fish intestinal histomorphometry and its association with growth performance. Information obtained from this study is thought to help guide fish farmers on NLP inclusion levels as a feed additive to Nile tilapia feeds for better growth performance in attaining the higher body weights of fish.

2.2 Materials and methods

2.2.1 Study area and ethical statement

The study was conducted at the Edward Moringe campus of Sokoine University of Agriculture, in Morogoro municipal. Located at 6°49′15″ S, 37°39′40″ E with an elevation of 504 m above sea level and the mean annual temperature is 24.3 °C. All experimental procedures were performed according to the guidelines for animal experimentation approved by the Research Ethical Committee of the Sokoine University of Agriculture (Ref no: SUA/DPRTC/R/186/63).

2.2.2 Identification, collection, and preparation of plant leaves

The neem plant was identified and authenticated by plant taxonomists in the Department of Crop Science and Horticulture, Sokoine University of Agriculture (Figure 1). Neem leaves were collected from SUA's main campus. This was done during the peak of the dry season on a day at around 13:00 hours when there was no moisture around the leaves. The leaves were washed and dried under shade at room temperature for two weeks. Then, it is ground into a fine powder using a laboratory mill machine (Christy Hunt grinder, Christy Hunt Engineering Ltd Atlas works, Colchester, Essex CO6 2EP England, Serial number 19911) and filtered using a 1.0 mm sieve. The obtained NLP was stored at room temperature in a dry laboratory container, ready to be incorporated in Nile tilapia commercial feeds at various doses.

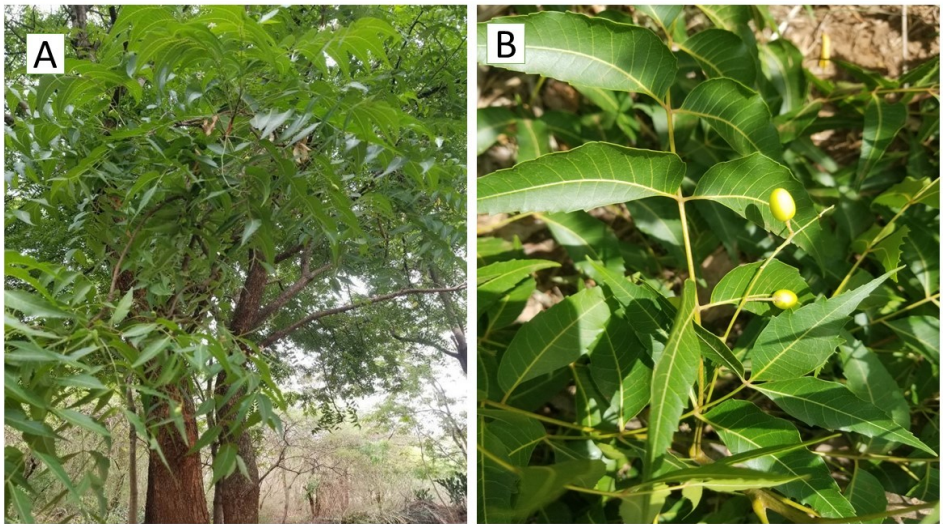

Figure 1. Neem tree on habitat in SUA farm (A): Neem leaves on closer view (B)

2.2.3 Phytochemical screening and proximate analysis

Phytochemical screening of plant samples was done at the Department of Chemistry and Physics, Sokoine University of Agriculture (Table 1). Proximate analysis of Nile tilapia commercial feed for fingerlings, NLP, and individual sample treatments (1 gKg^{-1}, 4 gKg^{-1}, and 8 gKg^{-1} feed) was done at the Department of Animal, Aquaculture and Range Sciences, Sokoine University of Agriculture (Table 2).

2.2.4 Treatment preparation and feeding regimes of Nile tilapia fingerlings

The NLP was incorporated in fish feed (Nile tilapia Commercial Feed for Fingerlings 39.73% crude protein (CP) pellet with 1mm size, from Koudijs International B.V). They were mixed at various doses of 1 g, 4 g, 8 g, and 0 g per Kg of feed. Fish were fed 5% of their body weight daily at 09:00 hours and 16:00 hours throughout the experimental period. Rations were modified, after measuring new body weights every 15 days.

2.2.5 Experimental Design and Fish Management

Mixed-sex Nile tilapia fingerlings weighing between 3 g and 10 g were purchased from the SUA-Aquaculture unit and were reared at the SUA-Aquaculture unit. The sample size of 180 fingerlings was used and determined by using G-Power software version 3.1.9.7 (Charan and Kantharia 2013). Fish were acclimatized for 14 days, before the start of the experiment. During this period, they were fed uniform Nile tilapia commercial feed. The experiment was done for 90 days, from August to October 2023, using two groups, the treated and the control group. The treated groups received NLP at concentrations of 1 g, 4 g, and 8 g for each kilogram of Nile tilapia commercial feed, and the control group was fed Nile tilapia commercial feed only. Each treatment and control group were replicated three times. The 180 fingerlings were randomly distributed and raised in 12 Clear-water Recirculation Aquaculture System (RAS) tanks, each with a stocking density of 15 fish per 100L

tank. Daily monitoring of physicochemical water parameters was done using (Hanna HI 98194 Multiparameter). Dissolved Oxygen (DO) ranged from 6.1 mgL^{-1} to 7.4 mgL^{-1}, Temperature ranged from 25.12 °C to 26.55 °C, pH ranged from 6.34 to 8.07, and Total Dissolved Solutes (TDS) ranged from 21 mgL^{-1} to 28 mgL^{-1}. Complete emptying and washing of tanks were done once each week. When necessary, depending on water turbidity siphoning, cleaning of the tanks, and water exchange, were done up to three times a week to bring water parameters into a normal state.

2.2.6 Growth Performance Indices

For every 15 days of the experimental period, growth performance indices were measured: Weight (g) using electronic beam balance, Standard Length (cm), and Total Length (cm) using a mathematical ruler placed on a wooden board to support the fish when measured. Total Length (TL) was measured from the mid tip of the snout to the end of the caudal fin, while Standard Length (SL) was measured from the tip of the snout to the beginning of the caudal fin. These measurements and other growth performance parameters were recorded in an Excel sheet. They were used to calculate Weight Gain (WG), Percent Weight Gain (PWG), Daily Weight Gain (DWG), Specific Growth Rate (SGR), Survival Rate (SR), Feed Conversion Ratio (FCR), and Conditional Factor (K) as described by (Alhassan et al. 2018; Kapinga et al., 2018). That is, WG (g) = (Final Weight - Initial Weight), PWG = [(WG)/(Initial Weight)] x 100, DWG ($gday^{-1}$) = WG/Days of experiment, SGR ($\%day^{-1}$) = [(Ln Final Weight - Ln Initial Weight)/(Days of experiment)] x 100, SR (%) = [(Final number of fish)/(Initial number of fish)] x 100, FCR = Feed intake (g)/WG (g), and K = [Mean Weight of Fish (W)/ Total Length (TL^3)] x 100.

2.2.7 Intestinal Histomorphometry

Fish were euthanized by being introduced into a beaker filled with three-quarters of distilled water and mixed with four drops of clove oil as

previously described (Mbugani et al. 2022). One fish was sampled per replicate at each period of day 0, 3, 30, 60, and 90 post-exposures, giving a total of 60 fish used for morphometric parameters. Intestinal samples were collected, and fixed in Bouin's solution. Then proximal intestine was dissected starting from the end of the pylorus region of the stomach to the beginning of the spiral intestine (Pirarat et al. 2011). The middle intestine was dissected from the start to the end of the spiral region, while the distal intestine was dissected from the region beyond the spiral portion up to 2cm before the anus (Pirarat et al. 2011). Three portions of proximal, middle/spiral, and distal intestines were cut 2 cm each. The tissues were then dehydrated in a series of graded ethanol, cleared using xylene, and embedded in paraffin wax. Tissues were sectioned at a size of 3 µm using a microtome (Baird and Tatlock-London LTD, Chadwell Heath, Essex, England. Ref. No 368065/2) and stained with hematoxylin and eosin (H&E). The stained tissue sections were then examined using a BH-2 light microscope linked with an Olympus camera for capturing microscopic images. The average of 10 highest and straight intestinal villi per intestinal segment per slide was measured from the middle of the villi top to the base of lamina propria (Figure 2). This was done using an ocular micrometer at 20X objective lens with a magnification factor of 4.18 µm.

2.2.8 Statistical analyses

Gathered data were converted into machine-readable data sets using Excel software, and tested for normality and equal variance by Kolmogorov–Smirnov and Levene's test respectively. Then the data analysis for statistical significance of means was done using Analysis of Variance (One-way ANOVA) and pairwise comparison of means was done by post hoc Tukey analysis. All the analyses were done using IBM SPSS software package version 25 for Windows, and a P-value < 0.05 was considered significant.

2.3 Results

2.3.1 Growth performance, feed utilization, and survival rate

The inclusion of NLP at 4 gKg^{-1} feed had a significant difference ($P < 0.05$) from the control and the rest of the treated groups regarding WG, PWG, DWG, and SGR. There was no significant difference in conditional factor (K) and FCR in both treatment and control groups throughout the experimental period. However, the conditional factor had an insignificant increase ($P > 0.05$) from the control group up to the dose of NLP 4 gKg^{-1} feed. While the dose of NLP at 8 gKg^{-1} feed had the lowest conditional factor (1.89). The inclusion level of NLP 4 gKg^{-1} feed had the lowest FCR (1.63), while the dose of NLP at 8 gKg^{-1} had the highest FCR (1.69). Survival rate (SR) had a significant difference ($P < 0.05$) at a dose of NLP at 8 gKg^{-1} feed (73.3%) in comparison to the control group (64.4%), Still, there was no significant difference among the treated groups (Table 3).

2.3.2 Intestinal histomorphometry

There was a significant difference ($p < 0.05$) in middle/spiral intestine villi height in the group that received 8 gKg^{-1} feed (141.51 µm) in contrast to the control group (166.36 µm). No significant difference was observed in the middle intestine among the treated groups. The proximal and distal portions of the intestine had no significant difference ($p > 0.05$) in villi height in both control and treated groups. However, there was an insignificant decrease ($p > 0.05$) in villi height as the doses of NLP increased compared to the control group (Figure 2; Table 4).

Table 1: **Screening for phytochemicals present in the leaves**

Parameters	Method Used
Flavonoids	Alkaline Reagent Test
Glycosides	Aqueous NaOH Test
Tannin	Braymer's Test
Saponin	Foam Test
Alkaloids	Mayer's Test
Carbohydrates	Molish's Test
Triterpenoids	Liebermann Burchard's Test

Table 2: **Proximate Analysis of the commercial feed and neem leaf powder**

Sample name	Dry Matter (%)	Ash (%)	Crude Protein (%)	Ether Extract (%)	Crude Fibre (%)	Moisture Content (%)
Tilapia Commercial Feed	93.76	9.83	39.73	4.86	5.23	6.34
Neem leaf powder	91.32	10.48	15.22	1.91	11.75	67.49
NLP 1 g/Kg Commercial Feed	92.04	9.07	39.71	3.38	8.63	7.96
NLP 4 g/Kg Commercial Feed	91.81	8.86	39.89	3.61	8.94	8.19
NLP 8 g/Kg Commercial Feed	92.15	8.92	39.93	3.35	8.77	7.85

Table 3: Growth performance of Nile tilapia fingerlings kept in clear-water RAS for 90 days

Growth Parameters	NLP 0 g/Kg feed	NLP 1 g/Kg feed	NLP 4 g/Kg feed	NLP 8 g/Kg feed	P-value
Initial Body Weight (g)	7.1 ± 0.03	7.2 ± 0.0	7.2 ± 0.06	7.1 ± 0.03	0.561
Initial Standard Length (cm)	5.1 ± 0.1	5.3 ± 0.03	5.0 ± 0.1	5.2 ± 0.2	0.371
Initial Total Length(cm)	6.9 ± 0.1	7.1 ± 0.03	6.8 ± 0.1	7.0 ± 0.2	0.448
Final Body Weight (g)	28.2 ± 1.1^{a}	28.6 ± 0.4^{a}	31.9 ± 0.8^{b}	28.5 ± 0.4^{a}	0.020
Final Standard Length (cm)	9.7 ± 0.1^{a}	9.6 ± 0.03^{a}	10.5 ± 0.1^{b}	9.7 ± 0.07^{a}	0.000
Final Total Length (cm)	12.1 ± 0.1^{a}	11.9 ± 0.07^{a}	13.2 ± 0.3^{b}	12.1 ± 0.09^{a}	0.002
WG (g)	21.1 ± 1.1^{a}	21.4 ± 0.4^{a}	24.7 ± 0.7^{b}	21.4 ± 0.3^{a}	0.019
PWG (%)	294.5 ± 15.8^{a}	296.7 ± 5.3^{a}	343.4 ± 7.1^{b}	299.5 ± 3.3^{a}	0.016
DWG (g/day)	0.23 ± 0.01^{a}	0.24 ± 0.00^{a}	0.3 ± 0.01^{b}	0.23 ± 0.00^{a}	0.019
SGR (%/day)	1.52 ± 0.05^{a}	1.53 ± 0.01^{a}	1.65 ± 0.02^{b}	1.54 ± 0.01^{ab}	0.021
SR (%)	64.4 ± 2.2^{a}	68.9 ± 2.2^{ab}	68.9 ± 2.2^{ab}	73.3 ± 0.0^{b}	0.067
FCR	1.67 ± 0.03	1.65 ± 0.03	1.63 ± 0.12	1.69 ± 0.04	0.923
Conditional Factor (K)	1.91 ± 0.01	1.93 ± 0.01	1.95 ± 0.01	1.89 ± 0.02	0.096

Parameters with different letter superscripts in a row differ significantly ($P < 0.05$) among different NLP doses. The data are presented as mean ± SEM ($n = 30$).

Table 4: Intestinal morphometry of Nile tilapia fingerlings for 90 days kept in clear-water RAS

Morphometric Parameters	NLP 0 g/Kg feed	NLP 1 g/Kg feed	NLP 4 g/Kg feed	NLP 8 g/Kg feed	P-value
Proximal Villi Height (µm)	236.03 ± 25.7	214.85 ± 18.04	200.86 ± 13.67	189.46 ± 9.4	0.327
Middle Villi Height (µm)	166.36 ± 8.5^{a}	152.66 ± 6.4^{ab}	146.53±3.0^{ab}	141.51 ± 2.2^{b}	0.045
Distal Villi Height (µm)	98.23 ± 6.5	91.01 ± 4.2	88.2 ± 2.97	85.17 ± 1.7	0.213

Parameters with different letter superscripts in a row differ significantly ($P < 0.05$) among different NLP doses. The values are presented as mean ± SEM ($n = 15$).

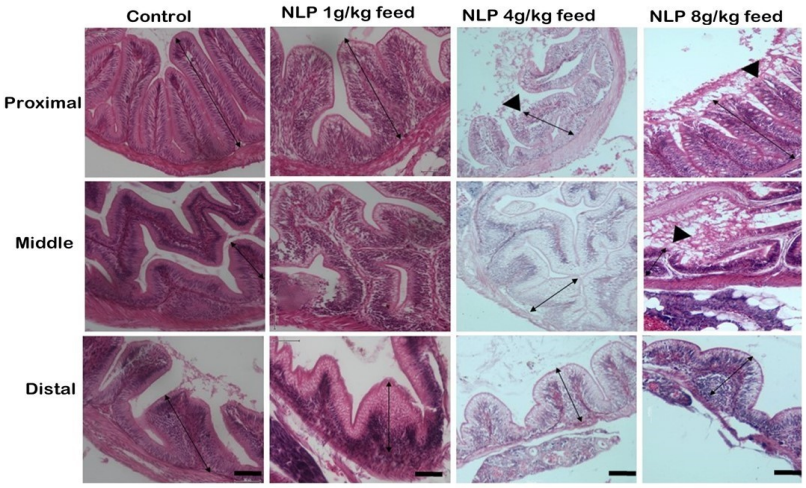

Figure 2: **Representative photomicrograph (Days 0-90). Intestinal tissue of Nile tilapia fingerlings post-exposure to NLP (0, 1, 4, and 8 g Kg**-1 **feed). Proximal, middle, and distal intestinal villi dimensions (double-headed arrow). Proximal, middle, and distal intestinal villi dimensions (double-headed arrow). There was a desquamation of intestinal epithelium (arrowhead), which was more pronounced in the middle intestine of the group that received NLP 8 gKg-1 feed. H&E, all columns with scale bar: 100 µm.**

2.4 Discussion

This study was conducted to assess the effect of NLP on the growth performance and intestinal histomorphometry of Nile tilapia fingerlings, aiming to thoroughly examine the effects of various doses of NLP used. The inclusion of different doses of NLP significantly influenced growth performance indices and intestinal histomorphometry. Specifically, the group administered NLP at 4 g Kg-1 feed exhibited notably higher values in WG, PWG, DWG, and SGR in tilapia fingerlings compared to those receiving NLP at 1 g Kg-1 feed, NLP at 8 g Kg-1 feed, and the control group. This contrasts with findings from other studies involving tilapia juveniles given NLP at 1 g Kg-1 feed and NLP at 8 g Kg-1 feed, where lower growth performance values were observed (Kapinga et al., 2018). Similarly, Nile tilapia juveniles that received NLP at 4 g Kg-1 feed demonstrated superior growth performance compared to the control group (Obaroh et al., 2014). Variances in growth performance among treated groups may be attributed to the presence of carbohydrates identified in phytochemical screening, as carbohydrates are known to enhance the growth performance of tilapia fingerlings (Jaravata et al., 2004). Additionally, the presence of saponins may have contributed to improved growth performance in Nile tilapia by positively influencing intestinal mucosal cell absorption (Obaroh et al., 2014). Furthermore, an increase in NLP doses from 1 g Kg-1 feed to 8 g Kg-1 feed corresponded with an increase in crude protein (CP) values according to proximate analysis. Previous studies have shown that elevated levels of crude proteins in feeds correlate with optimal growth performance and feed utilization in fingerlings (Ahmad and Abdel-Tawwab, 2011). However, growth performance declined at the NLP dose of 8 g Kg-1 feed, despite the highest CP content being recorded at 39.93%, potentially due to increased levels of tannin phytochemical, an anti-nutritional factor. Tannin, present at the NLP dose of 8 g Kg-1 feed, may have hindered digestive end-product absorption, as reported previously (Steiner and Encarnacão, 2010; Kim, 2017).

This study suggests that the early exposure of Nile tilapia fingerlings to NLP influences their adaptation to NLP doses. Administering NLP to newly hatched Nile tilapia resulted in improved reproduction control, better health, and superior growth performance without compromising the aquatic environment (Adjabi and Adjina, 2022). Similar observations of early adaptation to NLP doses and enhanced growth performance were noted in other fish species, including *Lates calcarifer* fingerlings and rainbow trout, when administered NLP doses ranging from 1 g Kg-1 feed to 5 g Kg-1 feed (Talpur and Ikhwanuddin, 2013; Abidin et al., 2022). NLP has also demonstrated efficacy in terrestrial mammals; for example, broiler chicks exposed to NLP doses ranging from 5 g Kg-1 feed to 15 g Kg-1 feed exhibited improved growth rates compared to the control group, with the NLP dose of 10 g Kg-1 feed yielding the highest performance (Mali et al., 2020). Similarly, sheep lambs fed NLP at 40 g Kg-1 dry matter feed for 90 days displayed significantly enhanced feed utilization, feed digestibility, and growth rates (El-Zaiat et al., 2022). Taking the data of this study together, we see that groups that received NLP had a positive effect on growth performance.

In terms of feed utilization, the FCR ranged from 1.63 to 1.69 across all groups in this study, with the group receiving NLP at 4 g Kg-1 feed exhibiting the best FCR of 1.63 alongside the highest growth performance indices. Generally, high growth performance correlates with low FCR values, a trend observed in studies involving aquatic and terrestrial animals administered NLP as a feed ingredient, where doses associated with high growth performance displayed low FCR values (Talpur and Ikhwanuddin, 2013; Kapinga et al., 2018; Mali et al., 2020). Additionally, the condition factor, indicative of growth performance, ranged from 1.89 to 1.95 in this study. As the condition factor exceeded one, it suggested that both treated and control fish groups exhibited isometric growth patterns, indicative of good health conditions and efficient feed utilization (Ayoade, 2011; Ighwela et al., 2011).

Survival rates (SR) increased with escalating doses of NLP, with the highest SR observed in the group receiving NLP at 8 g Kg-1 feed. This observation aligns with findings from a study on juvenile Nile tilapia, where the group administered NLP at 8 g Kg-1 feed achieved an 85% SR (Kapinga et al., 2018). Similarly, in other fish species such as *Lates calcarifer* fingerlings, the SR was higher in the group receiving NLP at 4 g Kg-1 feed but lower in the group receiving NLP at 5 g Kg-1 feed (Talpur and Ikhwanuddin, 2013). The increase in SR with higher doses of NLP may be attributed to the accumulation of phytochemical bioactive compounds present in neem leaves, which enhance the immunity of Nile tilapia fingerlings against stress (Kaur et al., 2020; Patel et al., 2021; Abu-Elala et al., 2023).

In Nile tilapia, growth performance cannot be solely explained by growth performance parameters but also by supporting evidence of the feed's effect on intestinal histomorphometry (Jaravata et al., 2004). Increased intestinal villi height observed in histomorphometry examinations indicates an expansion of the intestinal absorptive surface area for end-product nutrients (Jaravata et al., 2004; Pirarat et al., 2011). In this study, histomorphometry of the intestine was conducted across three sections: proximal, middle, and distal intestine. Throughout the experimental period, villi height remained longest in the proximal intestine, moderate in the middle, and shortest in the distal intestine in both control and treated groups, consistent with findings in other tilapia studies (Okuthe and Bhomela, 2021; Teixeira, 2023). However, the NLP dose of 8 g Kg-1 feed led to a significant decrease in villi height in the middle intestine compared to the control group. Similar decreases in villi height were noted in other studies, where high inclusion levels of plant-based canola meal or soybean meal in juvenile Nile tilapia feeds resulted in enteritis and reduced growth rates (Mohammadi et al., 2020; Ismail et al., 2019; Wang et al., 2022). The decreased villi height observed in this study may be attributed to elevated tannin levels and

prolonged retention of feed in the middle intestine due to its spiral nature, leading to enteritis and epithelial desquamation (Francis et al., 2001; Kim, 2017). Conversely, another neem product, neem seed protein hydrolysate (NSPH), maintained intestinal structure and increased villi height in the anterior intestine when included in Nile tilapia feeds, with the highest values recorded at 8% NSPH inclusion (Abdel-Rahman et al., 2023).

2.5 Conclusion

This is the first study to report on the effect of NLP on intestinal histomorphometry, with significantly shortened villi at a dose of NLP 8 g Kg^{-1} feed effect in the middle intestine. However, administration of NLP at a dose of 1 g Kg^{-1} feed up to 4 g Kg^{-1} feed has been shown to promote feed utilization and growth rate without affecting intestinal morphology.

Data availability statement

The data will be provided by the corresponding author when requested.

Conflict of interest

The authors declare no conflict of interest.

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CHAPTER THREE

MANUSCRIPT II

3.0 Histopathological effects of neem (*Azadirachta indica*) leaf powder on intestinal morphology of Nile tilapia (*Oreochromis niloticus*) fingerlings

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ABSTRACT

Neem (*Azadirachta indica*) leaf powder (NLP) has been used in juvenile Nile tilapia diets at inclusion levels of 2–8 g/kg feed to control reproduction. The powder is shown to induce degenerative histological changes in gonadal tissues. However, little is known on its effects on the gastrointestinal tract (GIT) of fish. This study aimed to evaluate the effects of NLP on intestinal morphology in Nile tilapia fingerlings. A total of 60 fish were randomly distributed in four groups (n = 15) and kept in a clear-water recirculation aquaculture system (RAS). The fish received

NLP at concentrations of 0 g, 1 g, 4 g, and 8 g for each kilogram of Nile tilapia commercial feed. The experiment was conducted for 90 days. Following euthanization by immersion in distilled water mixed with drops of clove oil, intestinal tissues were dissected and processed routinely for histopathological studies and immunostained for Tumor Necrotic Factor-alpha (TNF-α) and single-stranded DNA (ssDNA). Results showed that in all treated groups, there was an increased number of mucus-producing cells in the intestine i.e. proximal, middle, and distal when compared to the control group. In addition, epithelial desquamation was evident in the group that received NLP 8 g/Kg. Immunohistochemical analysis showed an increased expression of TNF-α and ssDNA in epithelium and pancreas in a dose-dependent manner. However, the severity of degenerative changes and expression of TNF-α and ssDNA in the epithelium decreased with time from day 30 to 90. In the pancreas, expression of TNF-α and ssDNA was detected in both early and long-term exposure, indicating that TNF-α has a fundamental role in the regulation of pancreas function. It can be concluded that, in short-term exposure, NLP induces histopathological changes in the fish intestine but the effects are reduced in long-term exposure.

Keywords: Neem plant, Intestine, Histomorphology, TNF-α, ssDNA

3.1 Introduction

The gastrointestinal tract (GIT) is the prominent site of intestinal absorption of digestive end-products, growth, and survival of fish (Jaravata et al. 2004; Okuthe and Bhomela 2021). Structurally it has the following tunics; the epithelium below it is a submucosa that starts inside the loose connective tissue to the point where both enterocyte linings contact each other and to the outermost layer is the tunica serosa (Kim 2017). Beyond the tunica serosa is located pancreatic tissue scattered along the whole part of the intestine and diffused within the mesenteries (Jaravata et al 2004; Genten et al 2009). The intestinal barrier when impaired by any injury, results in inflammatory cell infiltration through the expression of inflammatory cytokines such as tumor necrotic factor- alpha (TNF-α), and intestinal epithelial cells respond by secreting mucus as an integral immunological part of the fish intestine (Jutfelt 2011; Teixeira 2023). This damage in the intestinal tissue associated with TNF-α expression is usually induced directly or indirectly (Delgado and Brunner 2019). Direct damage of cells is mainly promoted by TNF-receptor 1 (TNFR1), and suppressing TNFR1 marks the end of TNF-α-induced apoptosis in the intestinal epithelial cells (Grabinger et al 2017). Meanwhile, TNF-receptor 2 (TNFR2) is associated with the induction of protective signals (Faustman and Davis 2013). Therefore, TNF-α has a key role in regulating the intestinal epithelial barrier (Turner 2009). Furthermore, TNF-α/TNFR-1 is associated with death receptors in the extrinsic pathway of apoptosis in the cytoplasmic part, while the intrinsic pathway of apoptosis is associated with DNA fragmentation taking place in the nucleus (Elmore 2007; Nowsheen and Yang 2012; Prokhorova et al 2015). This mostly occurs during the early stages of exposure associated with the shrinking of the cell, but pyknotic nuclei which is the most suggestive feature of apoptosis occur due to condensation of chromatin (Elmore 2007; Taatjes et al 2008; Nowsheen and Yang 2012). Therefore, cell death associated with the fragmentation of DNA can be detected by determining the expression of single-stranded DNA (ssDNA) which is

the best marker for apoptosis associated with DNA fragmentation (Frankfurt et al 1996; Ward et al 2008).

Neem (*Azadirachta indica*) leaf powder (NLP) as a phytomedicine has been included in juvenile Nile tilapia diets at inclusion levels of 2–8 g/kg feed to control reproduction, hence limiting overpopulation habit and stunted growth (Kapinga et al 2019). This was achieved due to NLP potency in exerting histological changes on gonadal tissues of Nile tilapia fish such as degenerative changes in the ovary and testis of juvenile Nile tilapia (Obaroh and Achionye-Nzeh 2011; Kapinga et al 2019). Due to this capability, neem leaves in Nile tilapia are given attention globally for having potent biologically active compounds with minimal side effects on the aquatic environment (Adjabi and Adjina 2022). Other histological effects were seen in *Labeo rohita* , a freshwater fish, on exposure to a sub-lethal concentration of neem leaf extract, which has been associated with mild to moderate inflammatory response in gill lamellae and shrunken secondary lamellae (Geetha et al 2019). On terrestrial animals, such as rats there have been reports of histological effects of neem leaves associated with the accumulation of lymphocytes and shrinkage in glomeruli on long-term exposure to neem leaf extract (Chibuike-Ikwuka et al 2020). There is increasing evidence of plant feed additives in fish feeds affecting intestinal histomorphology. Hence, studies on intestinal morphology are of enormous importance in the potency evaluation of NLP ingredients before incorporating them in fish diets (Kim 2017). Little is known about the NLP effect on the intestinal morphology in Nile tilapia fingerlings. Since fish intestinal morphology is strongly related to growth performance (Kim 2017; Teixeira 2023), it is paramount that the relationship between NLP and intestine structural morphology be studied. The hypothesis is that there is a strong association between fish intestine histomorphology and the feed ingested. Therefore, this study aimed to evaluate the effects of NLP on tilapia fish intestinal morphology using hematoxylin and eosin staining techniques and immunohistochemical analysis of TNF-α and ssDNA.

3.2 Materials and Methods

3.2.1 Study area

The study was conducted at the Sokoine University of Agriculture, in Morogoro municipal. Located at 6°49′15″ S, 37°39′40″ E with an elevation of 504 m above sea level and the mean annual temperature is 24.3 °C.

3.2.2 Identification, collection and preparation of plant leaves

The neem plant was identified and authenticated by plant taxonomists in the Department of Crop Science and Horticulture, Sokoine University of Agriculture (Figure 1). Neem leaves were collected from SUA's main campus. Then washed and dried under shade at room temperature for two weeks. Leaves were grounded into a fine powder using a laboratory mill machine (Christy Hunt grinder, Christy Hunt Engineering Ltd Atlas works, Colchester, Essex CO6 2EP England, Serial number 19911) fitted with a 1.0 mm screen. Then, NLP was stored at room temperature in a dry laboratory container, ready to be incorporated in Nile tilapia commercial feeds at various doses.

Figure 3: Neem tree in SUA farm (A): Neem leaves on a near view (B).

3.2.3 Phytochemical screening, plant leaves preparation and feeding regimes of Nile tilapia fingerlings

Phytochemical screening of plant leave samples was done at the Department of Chemistry and Physics, Sokoine University of Agriculture (Table 1). The NLP was incorporated in fish feed (Nile tilapia Commercial Feed for Fingerlings 39.73% crude protein pellet (CP) with 1mm size, from Koudijs International B.V). They were mixed at various doses of 1g, 4 g, 8 g, and 0 g per Kg of feed. Fish were fed 5% of their body weight daily at 09.00 and 16.00 hours throughout the experimental period. Rations were modified after measuring new body weights every 15 days.

3.2.4 Experimental design and animal management

Mixed-sex Nile tilapia fingerlings weighing between 3 g and 10 g were purchased from the SUA-Aquaculture unit and reared at the same unit. The sample size of 60 fingerlings was used, and fish were acclimatized for 14 days before the start of the experiment. During this period, they were fed uniform Nile tilapia commercial feed. The experiment was done for 90 days using two groups, the treated and the control group. The treated groups received NLP at concentrations of 1g, 4g, and 8g for each kilogram of Nile tilapia commercial feed, and the control group was fed Nile tilapia commercial feed only. Each treatment and control group were replicated three times. The 60 fingerlings were randomly distributed and raised in 12 Clear-water Recirculation Aquaculture System (RAS) tanks, each with a stocking density of 5 fish per 100L tank. Daily monitoring of physicochemical water parameters was done using (Hanna HI 98194 Multiparameter). Dissolved Oxygen (DO) ranged from 6.1 mg/L to 7.4 mg/L, Temperature ranged from 25.12 °C to 26.55 °C, pH ranged from 6.34 to 8.07, and Total Dissolved Solutes (TDS) ranged from 21 to 28. Complete emptying and washing of tanks were done once each week. When necessary, depending on water turbidity, siphoning, cleaning of the tanks, and water exchange, were done up to 3 times a week to bring water parameters into a normal state.

3.2.5 Tissue sampling and processing

Fish for intestinal histomorphology and immunohistochemistry were euthanized by immersion into a beaker filled with three-quarters of distilled water and mixed with four drops of clove oil (Mbugani et al 2022). One fish was sampled per replicate at each period of day 0, 3, 30, 60, and 90 post-exposures, resulting in a total of 60 fish used for intestinal histomorphology and immunohistochemistry. Intestinal samples were collected, and fixed in Bouin's solution. Then proximal intestine was dissected beginning from the end of the pylorus region of the stomach to the beginning of the spiral intestine (Pirarat *et al*., 2011). The middle intestine was dissected from the start to the end of the spiral region, while the distal intestine was dissected from the region beyond the spiral portion up to 2 cm before the anus (Pirarat *et al*., 2011). Three portions of proximal, middle/spiral, and distal intestine were cut each 2 cm. Thereafter, the portions were dehydrated in a series of graded ethanol, cleared using xylene, and embedded in paraffin wax. Tissues were sectioned at a size of 3 µm using a microtome (Baird and Tatlock-London LTD, Chadwell Heath, Essex, England. Ref.No 368065/2) for hematoxylin and eosin (H&E) and immunohistochemistry.

3.2.6 Intestinal histomorphology and immunohistochemical analyses

The prepared tissue sections were deparaffinized in xylene and rehydrated in decreasing the concentration of alcohol, washed in distilled water (3 x 5 minutes), and stained with H&E. Stained tissue sections were then examined using a BH-2 light microscope linked with an Olympus camera for capturing microscopic images.

For immunohistochemistry, initial processes were done as in H&E. After washing in distilled water, tissue sections were incubated with hydrogen peroxide block (ab64261) at room temperature (RT) for 30 minutes to block endogenous peroxidase activity. The cells were then washed with phosphate-buffered saline (PBS) for (3 x 5 minutes), followed by

incubation for 30 minutes at RT with two drops of goat serum at a dilution of 100:900 µl in PBS. Then, after rinsing the tissue sections in PBS, they were incubated with anti-mouse TNF-α primary antibody (ab1793-1001) overnight in a dark, humid container at 4 ℃, diluted at 10:990µl in PBS. Only PBS was added in the negative control slides instead of the primary antibody. The following day, after washing with PBS for (3 x 15 minutes), they were linked to a fluorescent donkey anti-mouse secondary antibody (ab150105-1001) diluted at 10:990 µl in PBS and incubated for 1 hour in a dark chamber. Next, the tissue slides were washed using PBS (3 x 5 minutes) and mounted using distyrene (polystyrene), a plasticizer (tricresyl phosphate), and xylene (DPX). Bounded antibodies were visualized in a darkroom using a BH-2 light microscope linked with an Olympus camera for capturing microscopic images. The same procedure was done by using an anti-mouse ssDNA primary antibody (ab313374-1001) alone as well as by mixing it with an anti-mouse TNF-α primary antibody (ab1793-1001) to check the effect of apoptosis in the nucleus (ssDNA) and cytoplasm (TNF-α).

3.2.7 Statistical analyses

Immunohistochemical expression of TNF-α via particle count was analyzed using Image J bundled with 64-bit Java 8. The particle counts were recorded in Excel software. Then analysis of data for statistical significance of means by Analysis of Variance (One-way ANOVA) using IBM SPSS software package version 25 for Windows. P-value < 0.05 was considered to be significant.

3.3 Results

3.3.1 Histomorphology

For the control group, all three portions of the intestine were in healthier morphology with the proximal and middle intestine possessing long, folded, and branched villi. In contrast, the distal intestine possessed short and unbranched villi. There was a healthier epithelium, which was

lined by columnar cells (enterocytes). Below the epithelium is a submucosa that starts inside the loose connective tissue to the point where both enterocyte linings contact each other, followed by tunica muscularis comprised of inner circular and outer longitudinal muscles and a serosal layer external to the tunica muscularis. Beyond the tunica muscularis is located pancreatic tissue scattered along the entire intestine and diffused within the mesenteries (Figure 2; control column).
The treated groups had an inflammatory reaction on early exposure (days 0–3) in the proximal, middle, and distal intestine epithelial portion of all treated groups; increased mucus-forming cells intertwined with inflammatory cells. There was increased severity as the dose increased associated with epithelium desquamation for the group that received NLP 8 g/Kg feed on the proximal intestine and was more intense in the middle intestine (Figure 2; the treated columns). However, on long-term exposure (day 30–90), there was complete epithelium recovery in all three portions of the intestines resembling the proximal, middle, and distal intestines of the control groups in Figure 2.

3.3.2 Immunohistochemistry

There was no expression of TNF-α or ssDNA in the tunica mucosa, submucosa, and tunica muscularis as well as in the pancreatic regions of all three intestinal portions of the control group from the start to the end of the experiment. However, for the treated groups, the early response (days 0–3) of the NLP post-exposure effect had a notably increased expression of TNF-α in the proximal, middle, and distal intestinal epithelium as well as in the pancreatic regions. This expression increased as the dose of NLP increased from a lower dose (1 g/Kg feed) to a higher dose (8 g/Kg feed). On long-term exposure (day 30–90) response, there was a remarkable decrease in the expression of TNF- α in the proximal, middle, and distal intestinal epithelium of all treated groups. However, there was a slight expression in pancreatic regions in all treated groups in the proximal and middle intestinal portions on (days 30–90). The expression of TNF- α upon

doing analytical procedures of TNF- α particle count Image J, all treated groups had a significantly higher expression ($p < 0.05$) in all intestinal portions when compared to the control groups on (days 0–3). However, there was a non-significant difference in all intestinal portions in both the treated and control groups ($p > 0.05$) on long-term exposure to NLP. Furthermore, there was immunopositivity detection for single-stranded DNA in nuclear portions of the intestinal epithelium and pancreatic regions in all three fish intestines in all treated groups in the early exposure (days 0–3) to NLP. However, it decreased on long-term exposure, and only slight expression was detected in the pancreatic regions (Figures 3, 4, 5, 6, 7; Table 2, 3).

Table 1: Screening for phytochemicals present in the leaves

Parameters	Method Used
Flavonoids	Alkaline Reagent Test
Glycosides	Aqueous NaOH Test
Tannin	Braymer's Test
Saponin	Foam Test
Alkaloids	Mayer's Test
Carbohydrates	Molish's Test
Triterpenoids	Liebermann Burchard's Test

Table 2: Expression of TNF- α in Nile tilapia fingerlings kept in clear-water RAS, early exposure 0-3 days

Particle Count of TNF- α	NLP 0 g/Kg feed	NLP 1 g/Kg feed	NLP 4 g/Kg feed	NLP 8 g/Kg feed	P-value
Proximal intestine	1214.0 ± 6.1^{a}	6670.33 ± 8.8^{b}	7231.33 ± 6.4^{b}	9075.67 ± 8.82^{b}	0.000
Middle intestine	1612.33 ± 7.9^{a}	7176.67 ± 8.81^{b}	8070.33 ± 5.4^{b}	9219.33 ± 10.48^{b}	0.000
Distal intestine	711.33 ± 5.8^{a}	6377.0 ± 5.77^{b}	7766.67 ± 5.7^{b}	8390.33 ± 5.21^{b}	0.000

Parameters with different letter superscripts in a row differ significantly ($P < 0.05$) among different NLP doses. The values are presented as mean ± SEM ($n = 6$).

Table 3: Expression of TNF- α in the Nile tilapia fingerlings long-term exposure 30-90 days kept in clear-water RAS

Particle	NLP 0 g/Kg	NLP 1 g/Kg	NLP 4 g/Kg	NLP 8 g/Kg	P-value

Count of TNF- α	feed	feed	feed	feed	
Proximal intestine	912.67 ± 51.2	962.0 ± 69.24	1104.33 ± 64.38	1173.0 ± 72.38	0.069
Middle intestine	1040.67 ± 30.18	1071.0 ± 139.4	1208.0 ± 97.12	1277.0 ± 63.38	0.290
Distal intestine	746.3 3± 114.9	812.0 ± 123.65	882 ± 84.86	880.67 ± 89.7	0.768

The values are presented as mean ± SEM (n = 9).

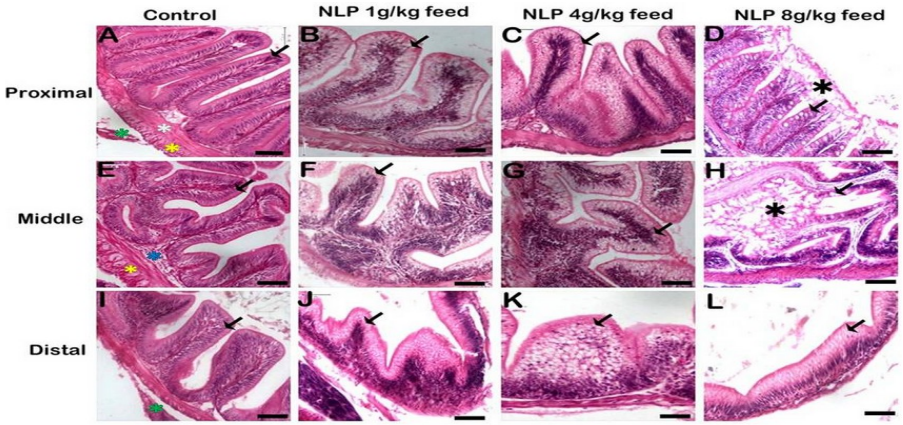

Figure 4: Representative photomicrograph of early exposure (days 0-3). Intestinal tissue of Nile tilapia fingerlings post-exposure to NLP (0, 1, 4, and 8 g/Kg feed). The control groups had healthier morphology in all three intestinal portions (A, E, and I; the epithelium (black arrowhead), submucosa (blue asterisk), inner circular muscle of tunica muscularis (white asterisk), outer longitudinal muscle of tunica muscularis (yellow asterisk), pancreas (green asterisk)). For the treated groups (B, C, D, F, G, H, J, K, and L) showed increased mucus production in the epithelial portion (black arrowhead). Epithelial desquamation (black asterisk) for a group receiving NLP 8 g/Kg feed on the proximal intestine was more intense in the middle intestine. H&E, scale bar: 100 µm.

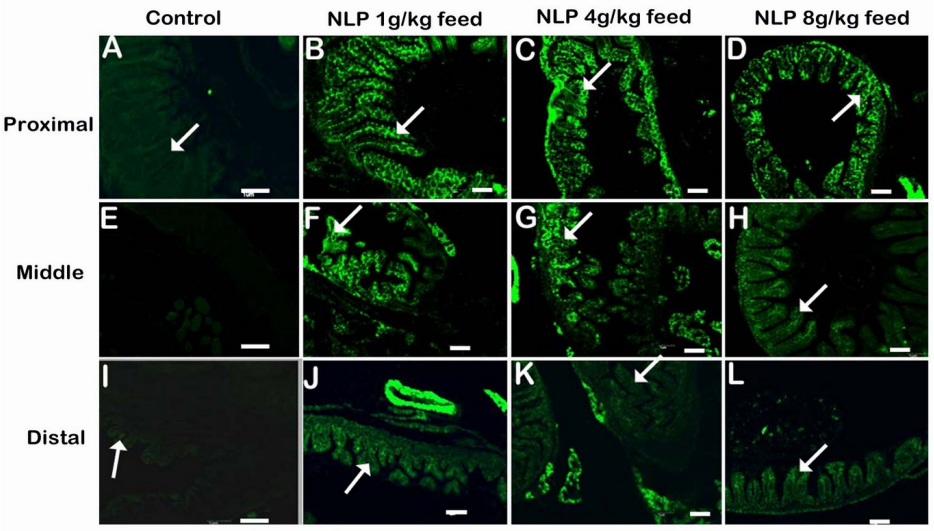

Figure 5: Representative photomicrograph of early exposure (days 0-3). Immunohistochemical expression of TNF-α on intestinal epithelium tissue of Nile tilapia fingerlings post-exposure to NLP (0, 1, 4, and 8 g/Kg feed). The control group shows no expression in all intestinal portions (A, E, and I). In treated groups (B, C, D, F, G, H, J, K, and L) increased expression is evident in all portions of intestinal epithelium (white arrow). Scale bar: 50 µm.

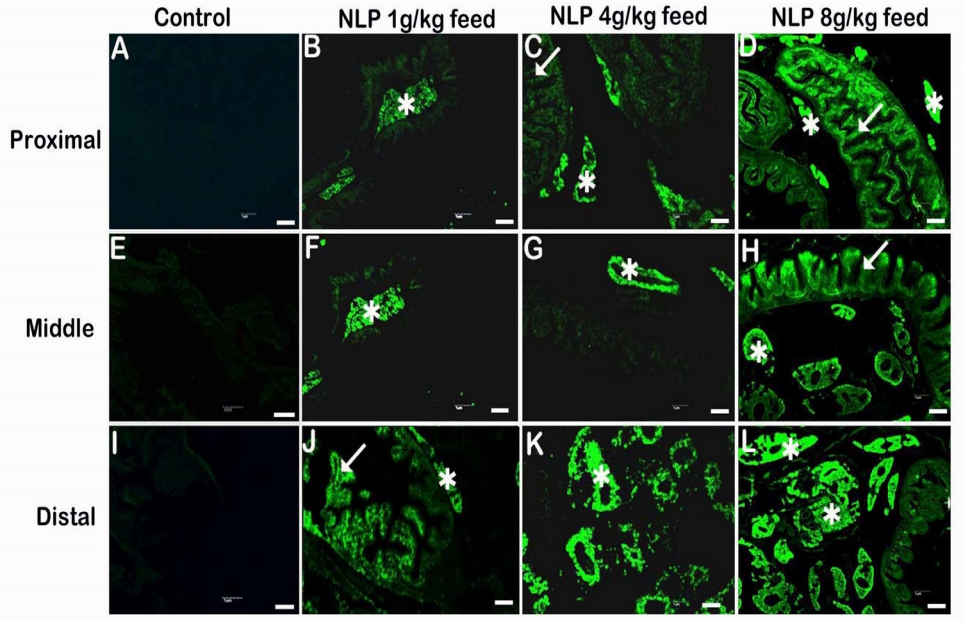

Figure 6: Representative photomicrograph of early exposure (days 0-3). Immunohistochemical expression of TNF-α on pancreatic tissue of Nile tilapia fingerlings located beyond the tunica serosa all along the proximal and middle intestine, and distal intestine post-exposure to NLP (0, 1, 4, and 8 g/Kg feed). The control group had no expression in all intestinal portions (A, E, and I). In treated groups (B, C, D, F, G, H, J, K, and L) there was increased expression in pancreatic tissue in all three portions of the intestines (white asterisk). Scale bar: 50 µm.

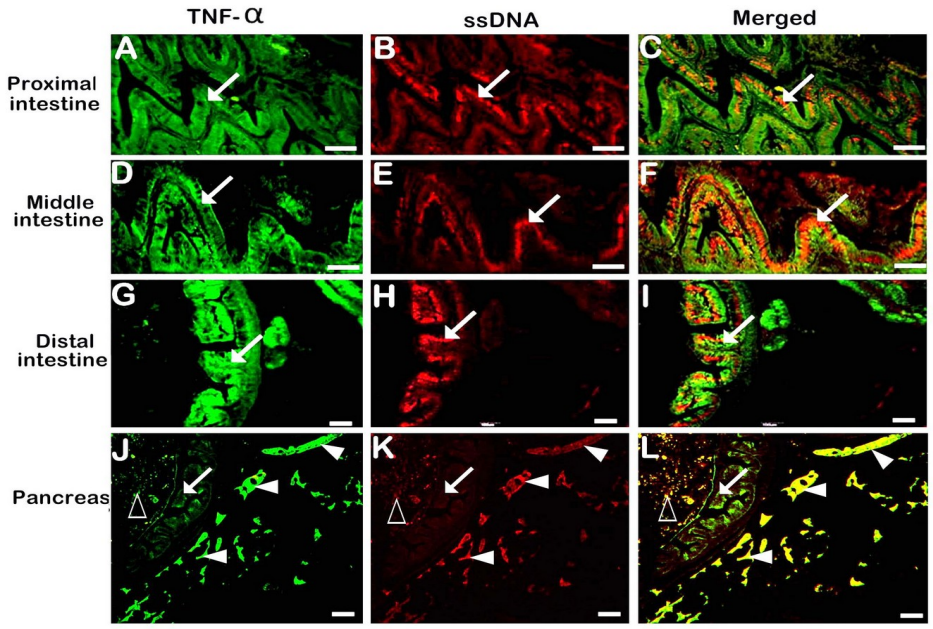

Figure 5: Double labeling immunofluorescence images showing TNF-α, and ssDNA immunoreactivity in intestinal tissues of Nile tilapia fingerlings post-exposure to NLP (0, 1, 4, and 8 g/Kg feed) for 0-3 days. The first column demonstrates labeling for TNF-α, (green; FITC), the second column for ssDNA (red; TRITC), and the third shows merged images. Positive immunoreactivity for TNF-α appears in the cytoplasm (white arrows) of the cells (A, D, and G), while those of ssDNA are in the nucleus (white arrows) in (B, E, and H). TNF-α and ssDNA do not co-localize in merged images (C, F, and I) in proximal, middle, and distal intestines. Expression of TNF-α and ssDNA is decreased in J, K, and L in the epithelium (white arrows) when the fish are exposed to NLP (0, 1, 4, and 8 g/Kg feed) for a long time (30-90 days). However, slight expression of TNF-α and ssDNA is still evident in the pancreas (white arrowheads) and in tissue sloughed off into the lumen (open arrowheads). Scale bar: 50µm.

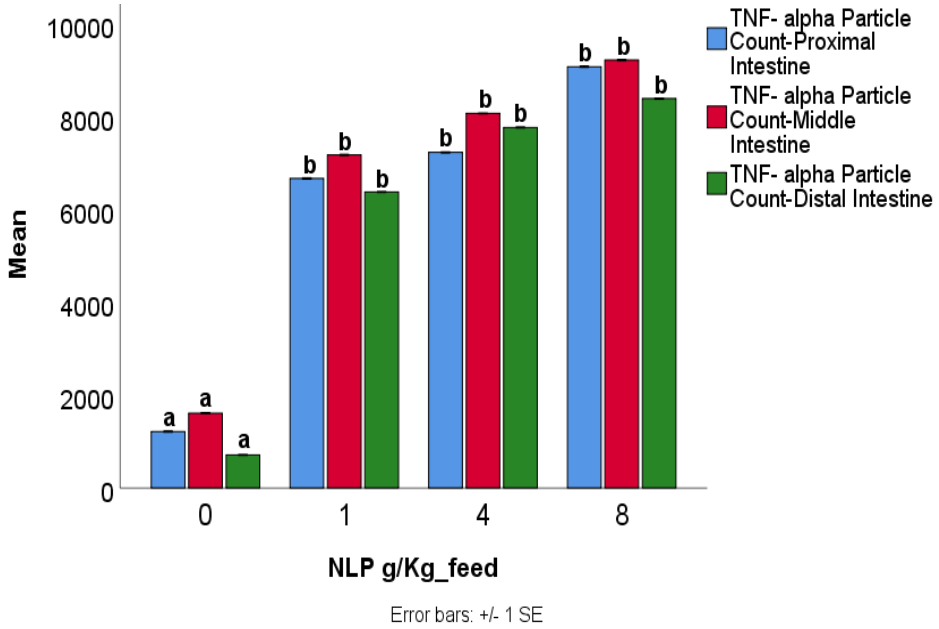

Figure 6: Early exposure (days 0-3). Effect of different concentrations of NLP on Nile tilapia; TNF-α particle count measurements of the proximal, middle, and distal intestine villi of the fish from different experimental groups. Bars with the same color with different letters superscript are significantly different at $P < 0.05$, using the Tukey post hoc test.

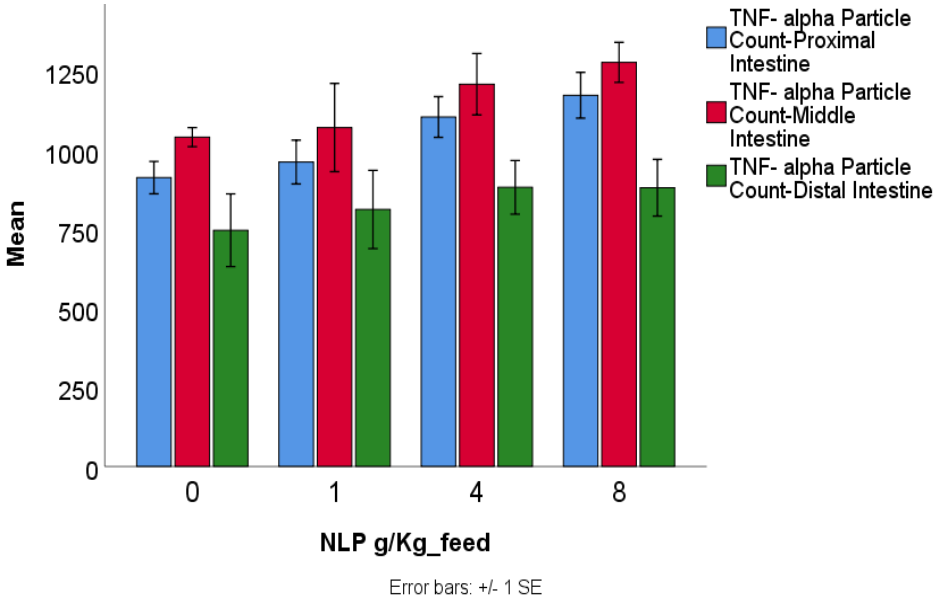

Figure 7: Long-term exposure (day 30-90). The non-significant effect of different concentrations of NLP on Nile tilapia; TNF-α particle count measurements of the proximal, middle, and distal intestine villi of the fish from different experimental groups.

3.4 Discussion

This study aimed to determine the effects of NLP on the intestinal morphology of Nile tilapia fingerlings using hematoxylin and eosin and immunohistochemistry for TNF-α and ssDNA. Results showed structural alterations in treated groups in which increased mucus production and epithelium desquamation (Figure 2), and immunoreactivity for TNF-α and ssDNA (Figure 3-7) were observed. Increase in intestinal mucus production, inflammatory cell infiltration, congestion, and epithelium desquamation were also detected in other fish species such as carnivorous fish and gilthead seabream after exposure to NLP (Estruch

et al 2018; Ngamkala et al 2020). When damaged by any injury, intestinal epithelial cells respond by secreting mucus as an integral immunological part of the fish intestine (Jutfelt 2011). The response is associated with a quick regeneration capacity crucial for the effective physiological functioning of the intestine (Delgado and Brunner 2019). Alteration in intestinal morphology is also associated with poor digestion and absorption of nutrients in fish, hence affecting growth performance (Leppkes et al 2014; Kim 2017). Moreover, damaged intestinal epithelium results in inflammatory cell infiltration by expressing inflammatory cytokines such as tumor necrotic factor- alpha (TNF-α) (Teixeira 2023). The TNF-α in intestinal epithelium performs a wide range of functions, such as inflammation regulation, apoptosis, and wound recovery (Leppkes et al 2014). It integrates the function of immune cells and intestinal epithelial cells during the inflammatory process (Delgado and Brunner 2019). Once a cell is damaged by any agent, including NLP, TNF-α produced by activated macrophages binds to cell surface receptors. The binding activates a series of caspases through membrane proteins, activating enzymes such as proteases and DNases. Enzymes cleave key cellular components, resulting in apoptosis, which is manifested by DNA fragmentation and degradation of cytoskeletal and nuclear proteins (Grabinger et al 2017). Therefore, cell death associated with the fragmentation of DNA can be detected by determining the expression of single-stranded DNA (ssDNA) which is the best marker for apoptosis associated with DNA fragmentation (Frankfurt et al 1996; Ward et al 2008). In this study, expression of TNF-α and ssDNA was found to increase with the increase in the dose of NLP in the first three days.

In a short time of exposure (0 to 3 days) to neem leaf extract, an increase in NLP doses was associated with increased expression of TNF-α and ssDNA, implying that neem leaves possess inflammatory activity (Dewanti 2011). In a long time (30 to 90 days), the leaf extract

showed a decreasing expression of TNF- and ssDNA, showing the anti-inflammatory potency of neem leaves. This coincides with other studies on long-term exposure to NLP (Ruslie and Darmadi, 2020). The anti-inflammatory potency of neem leaves was due to the presence of flavonoids (Table 1) (Nurmala et al 2020). Flavonoids possess anti-inflammatory activity by decreasing the expression of pro-inflammatory cytokines, such as TNF-α after prolonged exposure (Hoensch and Weigmann 2018; Al-Khayri et al 2022). The recovery effect on long-term exposure coincides with the study on juvenile Nile tilapia, where NLP 1g/L was found to alleviate pathological changes induced by lead toxicity on the gill, liver, and muscle tissues of fish (Abu-Elala et al 2023). Aqueous neem leaf extract exposed to African catfish (*Clarias gariepinus*) at a concentration of 3.5-7%, was also found to exert no pathological change on intestinal histomorphology, showing non-toxicity effect from neem leaves (Oniovosa et al 2017). However, in the pancreas, TNF-α and ssDNA were expressed in both early and long-term exposure to NLP. This coincides with previous studies on rats, which reported that early stages of cell injury due to foreign stimuli, the pancreatic acinar cells responded by producing TNF-α, which initiated a cascade of events leading to pancreatitis and apoptosis (Gukovskaya et al 1997; Malleo et al 2007).

3.5 Conclusion

The inclusion of NLP at doses 1-8 g/Kg feed has shown a histopathological effect on intestinal morphology with a significantly increased expression of TNF-α and ssDNA on the intestines of Nile tilapia fingerlings in the early stages of exposure. However, on long-term exposure, there was an adaptation to NLP doses on Nile tilapia fish due to flavonoid phytochemicals. Further research needs to be performed on the flavonoid phytochemicals in NLP that are responsible for inducing morphological changes in fish across the age group between juvenile and adult Nile tilapia.

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Ethical Considerations
All experimental procedures were performed according to the guidelines for animal experimentation approved by the Research Ethical Committee of the Sokoine University of Agriculture (Ref no: SUA/DPRTC/R/186/63).

Conflict of Interest
The authors declare no conflict of interest.

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CHAPTER FOUR

4.0 GENERAL DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

4.1 General Discussion

The aim of this study was to assess the effect of NLP on the intestinal histomorphology and growth performance of Nile tilapia fingerlings. Results showed that the inclusion of various doses of NLP significantly improved growth performance indices, with the group receiving 4 g/Kg feed exhibiting the best performance among all treated groups. These findings align with prior studies on juvenile Nile tilapia, where exposure to NLP resulted in notably higher growth rates compared to the untreated group. Specifically, the group exposed to 1 g NLP/Kg feed showed the highest growth rate among the exposed groups (Obaroh *et al*., 2014). Similarly, the inclusion of neem seeds in a protein hydrolysate form at 0 to 8% in Nile tilapia diets enhanced growth rate, with the highest improvement observed in the group exposed to 8% inclusion levels (Abdel-Rahman *et al*., 2023).

However, these findings contrast with a study on juvenile Nile tilapia, where slow growth was observed after exposure to 1 g and 8 g of NLP per Kg feed (Kapinga *et al*., 2018). This variation in the effectiveness of NLP doses across different studies may be linked to the age of Nile tilapia at exposure, whether in the fry stage, fingerlings, juveniles, or adult stage. Fingerlings in this study showed adaptation to NLP doses, with a higher inclusion level of 4 g NLP/Kg feed proving to be considerably more effective compared to 1 g NLP/Kg feed as observed in juvenile Nile tilapia (Obaroh *et al*., 2014). Additionally, exposure of NLP to Nile tilapia fry resulted in significant outcomes in managing overpopulation habits, promoting good growth rates, and enhancing the aquatic habitat (Adjabi and Adjina, 2022).

Furthermore, the group treated with NLP at 8g/Kg feed demonstrated significantly better survival compared to the control group, consistent with previous research on the Nile tilapia juvenile stage (Kapinga *et al*., 2018). However, survival at the end of exposure was lower for fingerlings (*Lates calcarifer*) exposed to NLP at 5 g/Kg feed (Talpur and Ikhwanuddin, 2013). The increased survival rate with higher NLP doses may be attributed to the presence of phytochemicals in neem leaves, which promote the innate immune system of Nile tilapia under various stress conditions (Kaur *et al*., 2020; Patel *et al*., 2021; Abu-Elala *et al*., 2023).

Intestinal histomorphometry provides valuable insights into the effects of specific fish diets on growth performance indices. High villi heights in the intestines indicate increased absorption area for digestion products. In this study, histomorphometry revealed that the NLP-exposed group at 8 g/Kg feed had reduced villi height compared to the unexposed group. This reduction in villi height is consistent with previous studies on plant-based diets, where excessive canola doses resulted in decreased villi height in Nile tilapia, leading to slower growth rates (Mohammadi *et al*., 2020). Similarly, soybean inclusion in juvenile Nile tilapia diets was associated with decreased villi height along the intestine (Ismail *et al*., 2019; Wang *et al*., 2022). Reduced villi height may be attributed to higher levels of tannin, an anti-nutritional factor in NLP, which can cause enteritis and hinder nutrient absorption, ultimately leading to stunted growth (Francis *et al*., 2001; Kim, 2017).

The effects of NLP on intestinal histomorphology included excess mucus production in all intestinal portions for the group exposed to 1 g/Kg feed. Excess mucus serves as a defense mechanism against harmful stimuli from NLP doses. Acute inflammatory responses, leading to epithelium sloughing in the proximal and middle intestines, were observed in groups exposed to 8 g/Kg feed. This inflammation was exacerbated by the branched nature of these intestines, making them

more susceptible to inflammation compared to the distal intestine (Kim, 2017). However, from day 30-90 of the experiment, there was regeneration of intestinal epithelium in the treated groups, indicating adaptation to NLP exposure and improved villi health, resulting in increased nutrient absorption and growth rates (Islam *et al*., 2021).

Throughout the experiment, the control group showed no changes in intestinal histomorphology or immunohistochemical expression of TNF-α and ssDNA. However, all exposed groups exhibited increased expression of TNF-α and ssDNA in all intestinal portions on days 0–3, which decreased with long-term exposure. Elevated TNF-α expression is associated with intestinal epithelium erosion and increased sloughing, consistent with the observed histomorphological changes. The increased mucus secretion also correlates with TNF-α expression, as TNF-α controls mucus secretion in the epithelium. These findings are supported by research on rat gastrointestinal tracts, which showed elevated TNF-α expression in groups exposed to neem leaf extract, indicating its inflammatory potential (Dewanti, 2011). However, contrary findings were observed in recent studies on rats exposed to neem leaf extract, where TNF-α expression decreased, suggesting the anti-inflammatory properties of neem leaves (Ruslie and Darmadi, 2020). This aligns with the present study's findings on long-term NLP exposure, where TNF-α expression decreased, likely due to the anti-inflammatory activity of flavonoids present in NLP (Nurmala *et al*., 2020; Al-Khayri *et al*., 2022).

4.2 Conclusion

The investigation into the inclusion of NLP as a phytomedicine feed additive in Nile tilapia feeds yielded noteworthy results, impacting both intestinal histomorphology and the growth performance of Nile tilapia fingerlings. This study marks the pioneering effort to document the effects of NLP on the intestinal histomorphology and growth

performance of Nile tilapia fingerlings. Notably, in terms of histomorphometry, a notable effect was observed in the middle intestine, characterized by shortened villi at a dosage of 8 g of NLP per kg of feed. Additionally, NLP dosages ranging from 1-8 g/kg of feed exhibited histopathological effects, alongside a significant increase in the expression of TNF-α and ssDNA in the intestines of Nile tilapia fingerlings during early exposure stages. However, this effect diminished with long-term exposure, resulting in similar outcomes between the treated and control groups. Conversely, concerning growth performance, all inclusion levels of NLP outperformed the control group, with a significant impact observed at the 4 g/kg feed dosage. Despite the frequent handling stress experienced by the fish due to tank cleaning and water exchange during turbid conditions in the rainy season, there was notable immune protective support observed, particularly evident in the enhanced survival rate of treated fish compared to the control group, with a significant effect noted at the 8 g/kg NLP dosage.

4.3 Recommendation

This study suggests inclusion levels of neem leaf powder (NLP) into the feeds of Nile tilapia fingerlings at a dosage ranging from 1 to 4 g/kg of feed during early growth stages to enhance weight gain while maintaining healthy intestinal morphology. However, it is essential to extend investigations into the effects of NLP on intestinal histomorphology in juvenile and adult Nile tilapia. Additionally, exploring the impact of NLP on other gastrointestinal organs like the spleen and liver is crucial to understanding the full scope of anatomical and physiological changes. Future research should focus on identifying the specific flavonoid phytochemicals within NLP responsible for morphological alterations in fish across different developmental stages. Furthermore, examining how the protective mechanisms of NLP evolve with increasing dosage levels is necessary for a comprehensive understanding of its effects.

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APPENDICES

Appendix 1: Ethical Clearance

STATEMENT OF RESEARCH ETHICAL APPROVAL

1. This project has been considered and has been **Approved/~~Not Approved~~** by the Department/College Research and Publication Committee, Department/College/Unit

   The proposal is relevant. The study will use fish as experimental units all ethical issues with regard to humane use of experimental animals has been taken care in the proposal specifically methodology section.

   Signature: ..... Name: **Alexander Mzula** Date: **2nd September 2023**
   (Chairperson, Research & Publication Committee)

2. This project has been considered and has been **Approved/~~Not Approved~~** by the Ethical Committee, DPRTC

   Signature: ..... Name: ALEXANDER MWANDI Date: 4th SEPT. 2023
   (Chairperson, Ethics Committee, DPRTC)

2. This project has been considered and **Approved/~~Not Approved~~** by the Committee responsible for Research and Publication, Sokoine University of Agriculture

   Signature: ..... Name: PROF. E.D. KARIMURIBO Date: 04-9-2023
   (Chairperson, SRPC)

   Director
   Postgraduate studies, Research,
   Technology Transfar and Consultancy
   Sokoine University of Agriculture
   P. O. Box 3151, Morogoro
   TANZANIA

SECTION P: FOR OFFICIAL USE

(i) APPROVAL

Date received : Click here and arrow to enter a date. 02nd Septmber 2023.	Received by: Click here to type names. MARTHA E. MOSHI
Date of approval: Click here and arrow to enter a date. Name: PROF: ESRON D. KARIMURIBO Title: DIRECTOR. Approving authority in capital letters (example: SRPC, Departmental /College/Centre R&PC **Click here to enter the name of approving authority.**	Approval reference number: SUA/DPRTC/R/186.163 Click here to enter number. Approval is valid from Director Postgraduate studies, Research, Technology Transfar and Consultancy Sokoine University of Agriculture P. O. Box 3151, Morogoro TANZANIA Click here and arrow to enter a date. 04.09.2023 To: Click here to enter a date.

*All undergraduate studies shall be evaluated and/or approved by the College/Centre R&PC and Reports submitted to the chair Research Ethica Committee, DPRTC

(ii) NOT APPROVED

☐The applicant is required to revise the application by addressing reviewer's concerns
(Reviwer's comments are provided to the applicant)

☐Other reasons (Describe briefly)

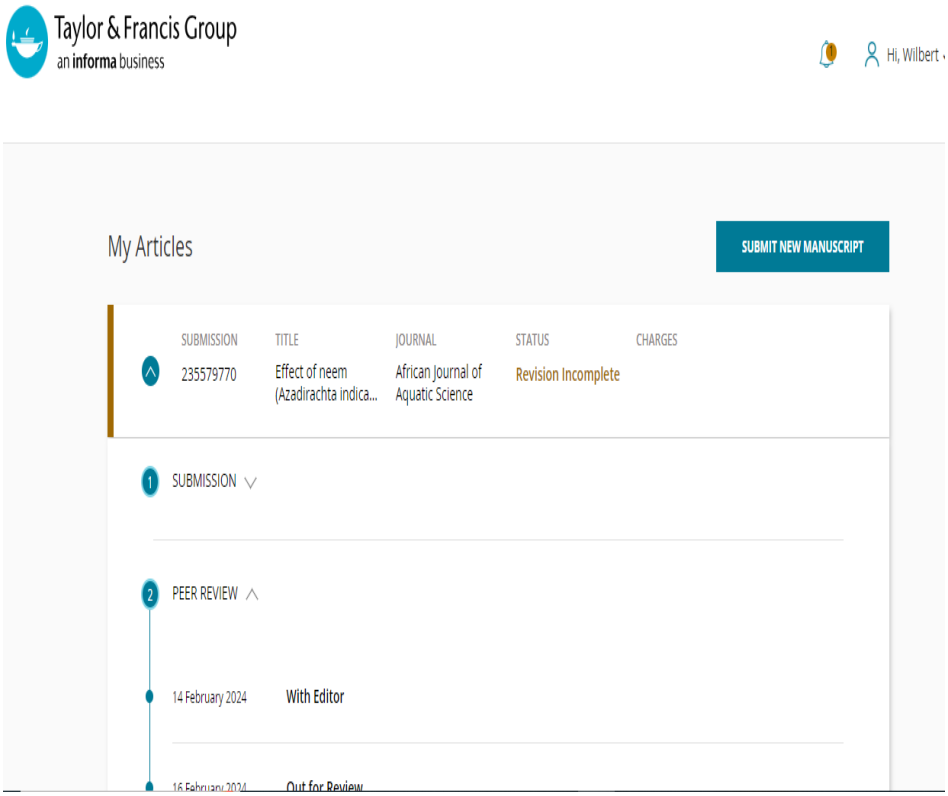
Appendix 2: Plagiarism report

EFFECT OF NEEM (AZADIRACHTA INDICA) LEAF POWDER ON INTESTINAL HISTOMORPHOLOGY AND GROWTH PERFORMANCE IN NILE TILAPIA (OREOCHROMIS NILOTICUS) FINGERLINGS

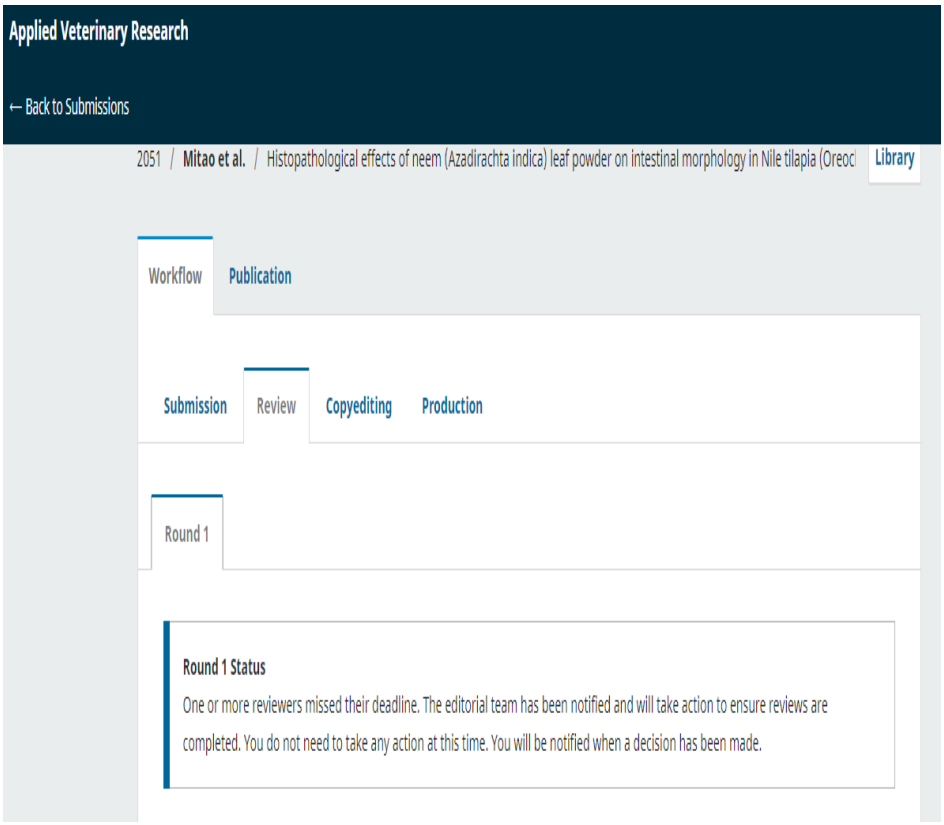
ORIGINALITY REPORT

17%	13%	12%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

Appendix 3A: Manuscript no.1 under review status



Appendix 4B: Manuscript no.2 under review status



Kuhusu Tasnifu Hii

Utafiti huu ulilenga kubainisha vifaranga wa samaki wa Sato walipokea unga wa majani ya mwarobaini (NLP) kwa viwango vya 0 g, 1 g, 4 g, na 8 g kwa kila kilogramu ya chakula cha Sato na majaribio yalifanyika kwa siku 90. Kiasi cha NLP 4 g/Kg ya chakula uliongeza sana ongezeko la uzito, asilimia ya ongezeko la uzito, ongezeko la uzito kila siku, na kiwango maalum cha ukuaji ikilinganishwa na vikundi vilivyolishwa kwa NLP 1 g na 8 g/kg ya chakula na kundi la kudhibitiwa. Zaidi ya hayo, ikilinganishwa na kikundi cha udhibiti, kikundi kilicholishwa kwa NLP (Siku 0-3) kilionyesha kuongezeka TNF-α na ssDNA katika sehemu za utumbo. Hii ilionyeshwa na athari ya uchochezi katika sehemu za utumbo wa juu, utumbo wa kati, na wa chini. Lakini kwa mfiduo wa muda mrefu (Siku 30-90), vikundi vyote vilivyolishwa NLP katika sehemu zao tatu za utumbo zilipata urejesho wa epithelial uliohusishwa na kupungua kwa TNF-α na ssDNA. Kwa hitimisho, kulikuwa na mabadiliko kwa mfiduo wa muda mrefu kwa viwango vilivyotumika vya NLP kwa vifaranga wa samaki wa Sato, na kuongezwa kutoka gramu moja hadi 4 za NLP kwa Kg ya chakula hakuna athari mbaya katika utumbo na inakuza ukuaji wa samaki.