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Physiological and Immunological Responses of Nile Tilapia (*Oreochromis niloticus*) Fed Diets Enriched with *Vitex agnus-castus* Extract

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الاستجابات الفسيولوجية والمناعية لأسماك البلطي النيلي (*Oreochromis niloticus*) المغذاة على وجبات مدعمة بمستخلص نبات كف مريم (*Vitex agnus-castus*)

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Abstract:
This study investigated the effects of dietary supplementation with *Vitex agnus-castus* fruit extract on Nile tilapia (*Oreochromis niloticus*). Two hundred and forty fish were randomly distributed into four groups and fed a basal diet supplemented with 0, 1, 2, or 4 g/kg of extract for 60 days. Results indicated that fish fed 2 and 4 g/kg exhibited significantly higher final weight, weight gain, and specific growth rate, alongside a lower feed conversion ratio compared to the control ($P < 0.05$). Activities of digestive enzymes including protease, amylase, and lipase, as well as antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, were significantly enhanced in all treated groups. The highest values for these parameters were observed at the 2 g/kg level. Furthermore, immune parameters including lysozyme activity, complement activity, and respiratory burst activity were significantly elevated in the treatment groups. The study concludes that dietary supplementation of *V. agnus-castus* extract at 2 g/kg optimally improves growth, enhances digestion, boosts antioxidant capacity, and strengthens immune function in *O. niloticus*.

Keywords: *Vitex agnus-castus*, *Oreochromis niloticus*, growth performance, antioxidant enzymes, immune response, phytobiotics.

الملخص
تناولت هذه الدراسة تأثيرات الإضافة الغذائية لمستخلص ثمار نبات كف مريم (*Vitex agnus-castus*) على أسماك البلطي النيلي (*Oreochromis niloticus*). تم توزيع مئتين وأربعين سمكة عشوائيًا على أربع مجموعات، حيث غُذيت على نظام غذائي أساسي مضاف إليه 0، 1، 2، أو 4 غرام/كغم من المستخلص لمدة 60 يومًا. أشارت النتائج إلى أن الأسماك التي غُذيت على 2 و 4 غرام/كغم أظهرت وزنًا نهائيًا، وزيادة في الوزن، ومعدل نمو نوعي أعلى بشكل معنوي، مع انخفاض في معامل التحويل الغذائي مقارنة بمجموعة السيطرة ($P < 0.05$). كما تم تعزيز أنشطة الإنزيمات الهضمية (البروتياز،

الأميليز، الليباز) والإنزيمات المضادة للأكسدة (السوبرأوكسيد ديسميوتيز، الكاتالاز، الجلوتاثيون بيروكسيديز) بشكل معنوي في جميع المجموعات المعاملة .سُجلت أعلى القيم لهذه المعايير عند مستوى 2 غرام/كغم .بالإضافة إلى ذلك، ارتفعت المعايير المناعية التي تشمل نشاط الليزوزيم، ونشاط المتممة، والنشاط الانفجاري التنفسي بشكل معنوي في مجموعات المعاملة . تخلص الدراسة إلى أن الإضافة الغذائية لمستخلص كف مريم بمقدار 2 غرام/كغم تحسن بشكل أمثل النمو، وتعزز الهضم، وتزيد القدرة المضادة للأكسدة، وتقوي الوظيفة المناعية في أسماك البلطي النيلي.

الكلمات المفتاحية: كف مريم(*Vitex agnus-castus*) ، البلطي النيلي(*Oreochromis niloticus*) ، أداء النمو، الإنزيمات المضادة للأكسدة، الاستجابة المناعية، المحفزات النباتية.

1. Introduction

The global aquaculture industry is paramount for securing food sustainability, with Nile tilapia (*Oreochromis niloticus*) standing as one of the most commercially significant farmed fish species worldwide (FAO, 2022). However, the transition toward intensive farming practices often subjects fish to various stressors, which significantly compromise their physiological state and immune function (Sarder et al., 2001; Dominguez et al., 2005). Such stressors increase susceptibility to devastating bacterial and viral pathogens, including *Aeromonas hydrophila* and Tilapia Lake Virus (TiLV), leading to substantial economic losses and prompting the prophylactic use of antibiotics (Pierezan et al., 2020; Zhao et al., 2022).

The immune system of Nile tilapia is a complex network of innate and adaptive components. The innate immune response serves as the first line of defense and is particularly crucial in fish, involving non-specific humoral factors and cellular responses (Sarder et al., 2001). Research has shown that these responses are highly sensitive to environmental fluctuations, such as changes in alkalinity and temperature (Dominguez et al., 2005; Zhao et al., 2022), as well as to the administration of vaccines and exposure to emerging contaminants like nanoparticles (Silva et al., 2009; Thummabancha et al., 2016). Furthermore, the pathogenesis of infections like *Photobacterium damselae* often involves immunosuppression, characterized by differential gene expression that weakens the host's natural defenses (Elbahnaswy & Elshopakey, 2020).

To mitigate these challenges, the search for sustainable, eco-friendly alternatives to antibiotics has intensified, focusing on the rise of antimicrobial resistance and environmental pollution (Salem & Terzi, 2019). Immunostimulants, including probiotics and phytogenic feed additives, have gained considerable attention. Probiotics, such as *Bacillus* species and *Lactobacillus plantarum*, have been shown to enhance growth performance and strengthen the immunological repertoire of Nile tilapia (Hamdan et al., 2016; Shija et al., 2023). Similarly, dietary supplementation with fermented *Spirulina platensis*, yeast (*Saccharomyces cerevisiae*), and herbal extracts has demonstrated significant improvements in antioxidant status and resistance against fungal and bacterial infections (Abdel-Tawwab et al., 2020; Sherif et al., 2024).

Phytogenic compounds, derived from plants like *Moringa oleifera*, *Viscum album*, and various herbal blends, exhibit a wide range of biological activities, including antimicrobial and anti-inflammatory properties (Park & Choi, 2012; Hassan et al., 2018; Abd El-Gawad et al., 2020). Among these botanical resources, *Vitex agnus-castus* L. (chaste tree) is renowned in traditional medicine. Its fruits are rich in bioactive compounds such as flavonoids (e.g., casticin), iridoid glycosides (e.g., aucubin, agnuside), and essential oils, which have demonstrated various pharmacological effects (Salem & Barkah, 2025).

While the efficacy of various feed additives and binders on the physiological and immune responses of *O. niloticus* is increasingly documented (Mohammady et al., 2025), specific research on the application of *V. agnus-castus* in tilapia culture remains scarce. Therefore, this study aimed to evaluate the effects of dietary supplementation with *V. agnus-castus* fruit extract on the growth performance, digestive enzyme activities, antioxidant status, and immune

responses of *O. niloticus*, providing a novel perspective on its potential as a functional feed ingredient in sustainable aquaculture.

2. Materials and Methods

2.1. Extract Preparation

Dried fruits of *Vitex agnus-castus* were purchased from a reputable herbal supplier. The fruits were ground into a fine powder, and the extract was obtained using aqueous-methanol (70:30 v/v) solvent extraction in a Soxhlet apparatus. The solvent was then evaporated under reduced pressure at 40°C using a rotary evaporator. The resulting crude extract was lyophilized and stored at -20°C until diet formulation.

2.2. Experimental Diets

A basal control diet (35% crude protein, 8% lipid) was formulated. The extract was incorporated into the basal diet at three different levels: 1, 2, and 4 g per kg of feed (denoted as VAC1, VAC2, and VAC4, respectively). The control diet (VAC0) contained no extract. All ingredients were thoroughly mixed, pelleted (2 mm diameter), air-dried, and stored at -20°C.

2.3. Fish and Experimental Design

A total of 240 clinically healthy Nile tilapia (*O. niloticus*) juveniles (15.2 ± 0.5 g) were obtained from a local hatchery. Fish were acclimatized to laboratory conditions for two weeks while fed the control diet. After acclimatization, they were randomly distributed into 12 fiberglass tanks (200 L each) representing four dietary groups in triplicate (20 fish per tank). The experiment was conducted in a recirculating aquaculture system with controlled water temperature (27 ± 1°C), pH (7.2 ± 0.2), and dissolved oxygen (>6 mg/L). Fish were fed to apparent satiation three times daily for 60 days.

2.4. Sampling and Analytical Procedures

At the end of the feeding trial, fish were fasted for 24 hours before sampling. Final body weight was recorded for growth performance calculation. Blood samples were collected from the caudal vein of three fish per tank using sterile syringes. Serum was separated by centrifugation (3000 × g, 10 min, 4°C) for immune parameter analysis. Liver and intestinal tissues from three fish per tank were dissected, snap-frozen in liquid nitrogen, and stored at -80°C for enzyme activity assays.

- **Growth Performance:** Weight Gain (WG), Specific Growth Rate (SGR), Feed Conversion Ratio (FCR), and Survival Rate (SR) were calculated.
- **Digestive Enzymes:** Activities of protease, amylase, and lipase in the intestine were measured using commercial kits.
- **Antioxidant Enzymes:** Activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in the liver were assayed using spectrophotometric methods.
- **Immune Parameters:** Serum lysozyme activity, alternative complement pathway activity (ACH50), and respiratory burst activity were measured.

2.5. Statistical Analysis

All data are presented as mean ± standard deviation (SD). Data were subjected to one-way analysis of variance (ANOVA) using SPSS software (v. 26.0). Differences between means were considered significant at $P < 0.05$ and determined by Duncan's multiple range test.

3. Results

3.1. Growth Performance and Feed Utilization

The growth performance and feed utilization parameters of *O. niloticus* fed the experimental diets are presented in Table 1. Final weight, WG, and SGR were significantly higher ($P < 0.05$) in groups VAC2 and VAC4 compared to the control group (VAC0). The best values were

observed in the VAC2 group. FCR was significantly lower in the VAC2 and VAC4 groups than in the control group. No significant differences were observed in survival rate among all groups.

Table 1. Growth performance and feed utilization of *O. niloticus* fed diets supplemented with *V. agnus-castus* extract.

Parameter	VAC0 (Control)	VAC1 (1g/kg)	VAC2 (2g/kg)	VAC4 (4g/kg)
Final weight (g)	42.5 ± 1.2c	45.1 ± 1.5b	49.8 ± 1.8a	48.3 ± 1.4a
WG (%)	179.6 ± 7.8c	196.7 ± 9.1b	227.6 ± 10.2a	217.8 ± 8.5a
SGR (%/day)	1.71 ± 0.05c	1.80 ± 0.06b	1.95 ± 0.07a	1.90 ± 0.05a
FCR	1.85 ± 0.08a	1.72 ± 0.07b	1.54 ± 0.06c	1.61 ± 0.07c
SR (%)	96.7 ± 3.3	98.3 ± 1.7	98.3 ± 1.7	95.0 ± 5.0

Values in the same row with different superscript letters are significantly different ($P < 0.05$). WG: Weight Gain; SGR: Specific Growth Rate; FCR: Feed Conversion Ratio; SR: Survival Rate.

3.2. Digestive Enzyme Activities

Dietary supplementation with *V. agnus-castus* extract significantly enhanced the activities of all tested digestive enzymes ($P < 0.05$, Fig. 1). The highest activities of protease, amylase, and lipase were recorded in the VAC2 group, which were significantly higher than those in the VAC0 and VAC1 groups. The VAC4 group also showed significantly higher enzyme activities than the control.

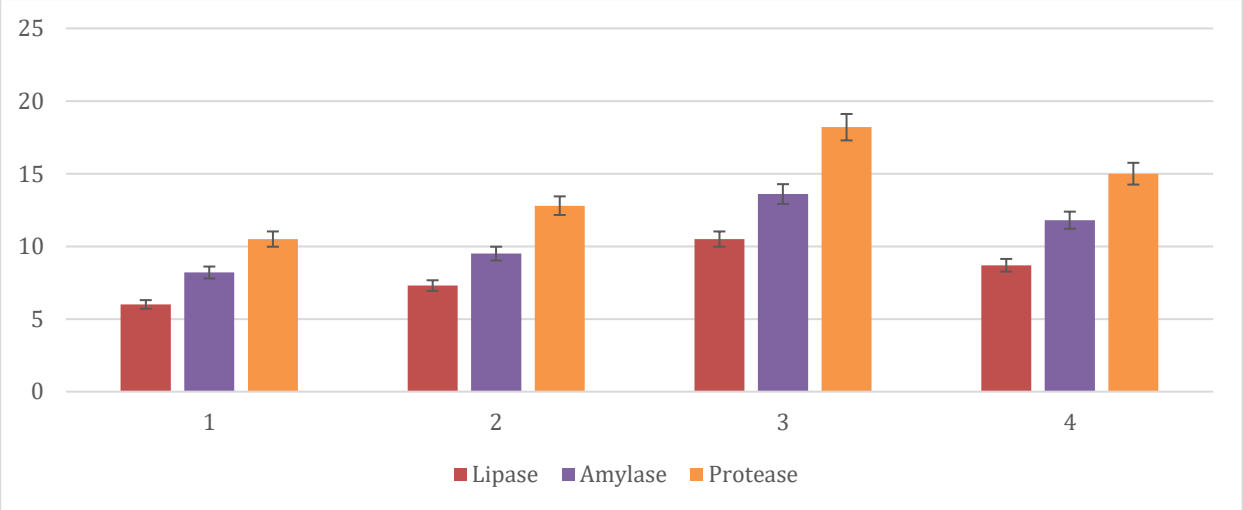

Fig. 1. Effect of *V. agnus-castus* extract on digestive enzyme activities in *O. niloticus*. **Bars with different letters are significantly different (P < 0.05),1;VAC0% ,2;VAC1%, 3;VAC2%;4,VAC4%.**

3.3. Antioxidant Enzyme Activities

Hepatic antioxidant enzyme activities are shown in Fig. 2. SOD, CAT, and GPx activities were significantly higher in all treatment groups compared to the control group ($P < 0.05$). The VAC2 group consistently exhibited the highest antioxidant enzyme activities among all groups.

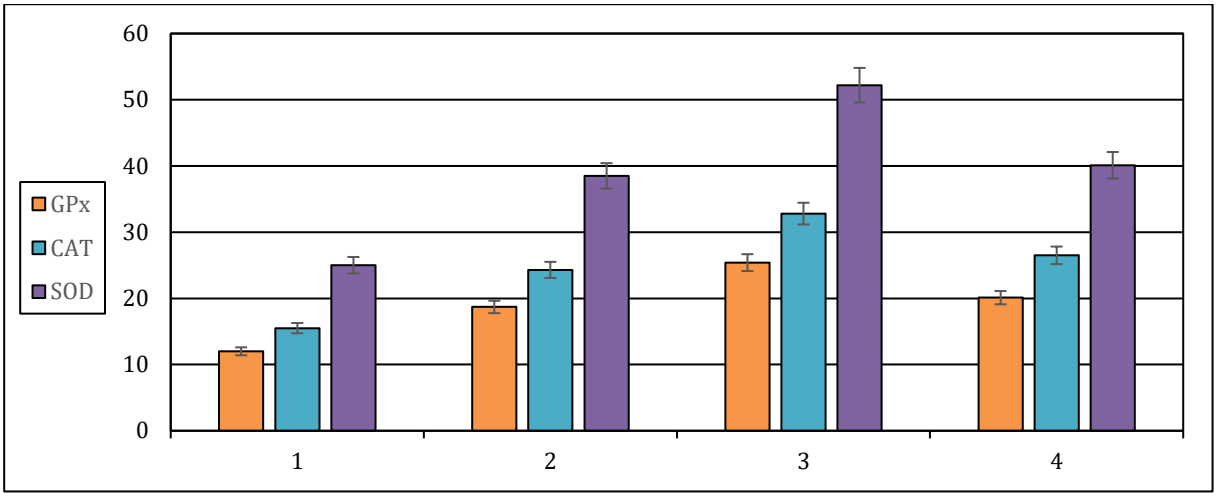

Fig. 2. Effect of V. agnus-castus extract on hepatic antioxidant enzyme activities in *O. niloticus*.

Bars with different letters are significantly different (P < 0.05).

3.4. Immune Parameters

The non-specific immune parameters of tilapia were significantly influenced by the dietary treatments (P < 0.05, Table 2). Serum lysozyme activity, ACH50, and respiratory burst activity were significantly elevated in the VAC1, VAC2, and VAC4 groups compared to the VAC0 group. The highest immune responses were generally observed in the VAC2 group.

Table 2. Immune parameters of *O. niloticus* fed diets supplemented with V. agnus-castus extract.

Parameter	VAC0 (Control)	VAC1 (1g/kg)	VAC2 (2g/kg)	VAC4 (4g/kg)
Lysozyme (U/mL)	85.2 ± 6.1c	105.7 ± 7.8b	132.5 ± 9.4a	121.8 ± 8.5a
ACH50 (U/mL)	32.5 ± 2.8c	41.8 ± 3.5b	52.6 ± 4.1a	48.3 ± 3.7a
Respiratory burst (OD)	0.35 ± 0.03c	0.48 ± 0.04b	0.62 ± 0.05a	0.55 ± 0.04ab
Values in the same row with different superscript letters are significantly different (P < 0.05).				

4. Discussion

The results of this study demonstrate that dietary supplementation with *V. agnus-castus* extract significantly enhances the overall physiological performance and immune status of Nile tilapia (*O. niloticus*). The observed improvements in growth performance and feed utilization (Table 1) are consistent with previous findings regarding the efficacy of phytogenic feed additives in aquaculture. For instance, the enhanced Growth Performance and Feed Conversion Ratio (FCR) align with observations by Sherif et al. (2024) and Hassan et al. (2018), who noted that natural supplements like fermented *Spirulina* and herbal blends can optimize nutrient assimilation. This growth-promoting effect is likely due to the presence of bioactive compounds like flavonoids and essential oils, which have been shown to modulate the gut microbiota and improve intestinal health (Hamdan et al. (2016); Kadak & Salem, 2020).

4.1. Digestive Enzymes and Growth Mechanisms

The significant increase in digestive enzyme activities (Fig. 1) provides a physiological explanation for the improved Specific Growth Rate (SGR). By stimulating the secretion of proteases and amylases, *V. agnus-castus* facilitates a more efficient breakdown of complex nutrients. This is further supported by Mohammady et al. (2025), who highlighted how dietary components and binders influence the digestive efficiency and physiological response in Nile tilapia. The slight reduction in efficacy observed at the highest dose (4 g/kg) suggests a "hormetic effect" or a physiological ceiling, where excessive concentrations of phytogenic compounds might lead to minor inhibitory actions or metabolic overload, a phenomenon often discussed in herbal supplementation studies (Salem et al., 2022).

4.2. Antioxidant Defense and Oxidative Stress

A key finding of this study is the potent antioxidant capacity of *V. agnus-castus* extract. Intensive aquaculture often leads to the overproduction of Reactive Oxygen Species (ROS), causing oxidative stress and cellular damage (Salem, 2025). The significant elevation in SOD, CAT, and GPx activities (Fig. 2) indicates a strengthened endogenous defense system. This mirrors the results of Abdel-Tawwab et al. (2020) and Sherif et al. (2024), who reported that supplements like yeast and *Spirulina* enhance the antioxidative status of Nile tilapia, thereby protecting them from environmental and biological stressors. The iridoid glycosides and flavonoids in the extract likely act as direct ROS scavengers and upregulate antioxidant gene expression (Taştan & Salem, 2021).

4.3. Immunomodulatory Effects

The immunostimulatory effects of the extract were evident from the enhanced lysozyme, complement, and respiratory burst activities (Table 2). These non-specific immune responses are the primary defense mechanisms in tilapia (Sarder et al., 2001). Our findings are in agreement with Park & Choi (2012), who demonstrated that mistletoe (*Viscum album*) extract enhances innate immunity in *O. niloticus*.

Furthermore, the boost in immunity observed here is crucial for resistance against common pathogens. For instance, Abd El-Gawad et al. (2020) and Zhao et al. (2022) emphasized that strengthening the innate immune system is vital for surviving challenges from *Aeromonas hydrophila* and environmental stressors like alkalinity. The bioactive compounds in *V. agnus-castus* appear to prime the immune cells, such as macrophages and neutrophils, leading to higher phagocytic activity and respiratory burst (Amhamed et al., 2023). This is particularly important given that viral pathogens like TiLV and bacterial infections can cause severe immunosuppression (Pierezan et al., 2020; Elbahnaswy & Elshopakey, 2020).

4.4. Practical Implications for Sustainable Aquaculture

The ability of *V. agnus-castus* to serve as a natural immunostimulant offers a promising alternative to antibiotics, addressing concerns over antimicrobial resistance (Salem & Terzi, 2019). Similar to the effects of probiotics like *Lactobacillus plantarum* (Hamdan et al., 2016), this plant extract provides a holistic approach to fish health—improving growth, protecting against oxidative damage, and reinforcing the immune barrier against pathogenic threats (Shija et al., 2023).

5. Conclusion and Recommendations

5.1. Conclusion

The findings of this study provide compelling evidence that dietary supplementation with *Vitex agnus-castus* fruit extract is highly beneficial for Nile tilapia (*Oreochromis niloticus*) culture.

As a multifaceted functional feed additive, it significantly improves growth performance and feed efficiency, likely through the modulation of digestive enzyme secretion and enhanced nutrient assimilation (Mohammady et al., 2025).

Beyond growth, the extract serves as a potent biostimulant that boosts the hepatic antioxidant defense system (SOD, CAT, and GPx), protecting the fish from the physiological rigors of intensive farming (Abdel-Tawwab et al., 2020; Sherif et al., 2024). Crucially, the reinforcement of innate immune responses—specifically lysozyme and respiratory burst activities—positions *V. agnus-castus* as a viable natural alternative to synthetic immunostimulants and antibiotics (Sarder et al., 2001; Hamdan et al., 2016). Based on the integrated analysis of growth, physiological health, and immunological vigor, the optimal inclusion level was determined to be 2 g per kg of diet, as higher concentrations did not yield proportional benefits.

5.2. Recommendations

Based on the comprehensive findings of this research, the following recommendations are proposed to bridge the gap between experimental results and commercial application:

Practical Application in Aquafeeds: *Vitex agnus-castus* extract should be integrated into commercial tilapia starter and grower diets at a concentration of 2 g/kg. This dosage is recommended to promote superior growth, optimize FCR, and provide a robust immunological "primer" against common aquatic pathogens.

Given the emerging threats of antimicrobial resistance (Salem & Terzi, 2019), farm managers should prioritize such phytogenic additives to reduce reliance on therapeutic chemicals.

Strategic Further Research:

1. **Pathogenic Challenge Trials:** Future studies must evaluate the protective efficacy of the extract through *in vivo* challenges with virulent strains such as *Aeromonas hydrophila* and Tilapia Lake Virus (TiLV), building on the pathogenesis frameworks established by Pierezan et al. (2020) and Zhao et al. (2022).
2. **Long-term & Meat Quality Assessment:** Investigations should extend to the full production cycle to determine the long-term effects of supplementation on fish health, fillet quality (sensory and nutritional profiles), and shelf-life.
3. **Molecular Mechanisms & Standardization:** Research is needed to isolate and standardize the specific active compounds (e.g., casticin, aucubin) responsible for the observed effects. Understanding the gene expression pathways involved in the fish's immune response will provide deeper mechanistic insights (Elbahnaswy & Elshopakey, 2020).
4. **Economic Scalability:** A detailed cost-benefit analysis is required to evaluate the economic feasibility of large-scale application in commercial aquaculture, ensuring that the health benefits translate into increased profitability for producers.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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