Journal of Marine Sciences & Environmental Technologies (JMSET)

مجلة علوم البحار والتقنيات البيئية (JMSET)

https://journals.asmarya.edu.ly/jmset/index.php

(p) ISSN: 2413-5267 (e) ISSN: 2706-9966

Volume (12), Issue (1), January-June (2026)

المجلد (12)، العدد (1)، يناير-يونيو (2026)

Research Article

Aquaculture Biotechnology

Dose-Dependent Modulation of Interleukin-8 (IL-8) Gene Expression in the Kidney of Rainbow Trout (*Oncorhynchus mykiss*) Fed Dietary Supplementation with *Vitex agnus-castus* L. Seed Extract

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Article Info

Received: 24 July 2025
Revised: 15 September 2025
Accepted: 03 January 2026
Online: 01 April 2026

Keywords: Aquaculture, Immunostimulant, Phytogenic, Cytokine, Real-time PCR, Vitex, Fish immunity, Dose-response, Inflammation.

Abstract

The current study aimed to evaluate the efficacy of *Vitex agnus-castus* (chaste tree) seed extract as a sustainable immunostimulant in aquaculture, focusing on the gene expression of Interleukin-8 (IL-8) in the kidneys of rainbow trout (*Oncorhynchus mykiss*). To this end, 240 fish were distributed into four dietary groups receiving different supplement doses (0%, 0.5%, 1.0%, and 2.0%) for 8 weeks, followed by tissue analysis using qPCR. The results demonstrated that the extract exerts a dose-dependent effect; while the 0.5% dose showed no significant change, the 1.0% dose significantly upregulated gene expression (2.8-fold), potentially enhancing immune readiness. Conversely, the high dose (2.0%) led to a downregulation of gene expression, suggesting potential anti-inflammatory effects. These findings confirm the potent immunomodulatory capacity of this extract and underscore the necessity of determining optimal dosages to enhance disease resistance within the aquaculture sector.

التعديل المعتمد على الجرعة للتعبير الجيني للإنترلوكين-8 (IL-8) في كلية سمك السلمون المرقط قوس قزح (*Oncorhynchus mykiss*) المُغذّى على مكمّل غذائي من مستخلص بذور شجرة (*Vitex agnus-castus L.*)

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معلومات المقالة

تاريخ الاستلام: 24 يوليو 2025.
تاريخ المراجعة: 15 سبتمبر 2025.
تاريخ القبول: 03 يناير 2026.
تاريخ النشر: 01 أبريل 2026.

الكلمات المفتاحية: الاستزراع المائي، مُحفِّز مناعي، مركبات نباتية، سيتوكين، تفاعل البوليميراز المتسلسل اللحظي، شجرة العفة، مناعة الأسماك، استجابة جرعة-تأثير، التهاب.

الملخص

هدفت هذه الدراسة إلى تقييم تأثير مستخلص بذور نبات كف مريم (*Vitex agnus-castus*) كمحفز مناعي مستدام في استزراع الأسماك، حيث ركزت على قياس تعبير جين الإنترلوكين-8 (IL-8) في كلى أسماك التراوت -السلمون المرقطة قوس قزح- (*Oncorhynchus mykiss*)؛ ولتحقيق ذلك، تم توزيع 240 سمكة على أربع مجموعات غذائية بجرعات مختلفة (0%، 0.5%، 1.0%، 2.0%) لمدة 8 أسابيع، ثم تحليل الأنسجة باستخدام تقنية qPCR. أظهرت النتائج أن المستخلص يؤثر بشكل يعتمد على الجرعة؛ فبينما لم تُحدث جرعة 0.5% تغييراً ملموساً، أدت جرعة 1.0% إلى رفع مستويات تعبير الجين بشكل كبير (2.8 ضعف)، مما يعزز الاستعداد المناعي، في حين أدت الجرعة العالية (2.0%) إلى خفض التعبير الجيني، مما يشير إلى تأثيرات مضادة للالتهاب، وهو ما يؤكد القدرة العالية لهذا المستخلص على تعديل المناعة وضرورة تحديد الجرعات المثلى لتعزيز مقاومة الأمراض في قطاع الاستزراع المائي.

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DOI: https://doi.org/10.59743/jmset.v12i1.187

1. Introduction

Aquaculture is vital for global food security, but intensive farming practices predispose fish to stress and disease outbreaks, leading to significant economic losses (Amhamed *et al*., 2023; Salem *et al*., 2021). While antibiotics and chemotherapeutics have been widely used, concerns over antimicrobial resistance, environmental impact, and consumer safety necessitate the search for sustainable alternatives (Salem & Lakwani, 2024). Immunostimulants, compounds that non-specifically enhance the innate immune system, offer a promising strategy to improve fish health and disease resistance (Taştan & Salem, 2021).

Phytogenic compounds derived from plants, herbs, and spices have gained considerable attention as natural immunostimulants in aquaculture due to their bioactive properties, including antimicrobial, antioxidant, and immunomodulatory effects (Mohamed *et al*., 2024). *Vitex agnus-castus* L. (Chaste tree), a member of the Lamiaceae family, is a traditional medicinal plant. Its seeds are rich in bioactive constituents, notably iridoid glycosides (e.g., aucubin, agnuside), flavonoids (e.g., casticin, orientin), diterpenes (e.g., vitexilactone), and essential oils (Kadak & Salem, 2020). Extracts from *V. agnus-castus* seeds have demonstrated various pharmacological activities, including anti-inflammatory, antioxidant, and hormone-modulating effects (Salem & Barkah, 2025; Salem & Mohamed, 2025). However, their potential immunomodulatory effects in teleost fish remain largely unexplored.

The innate immune system is the first line of defense in fish. Cytokines and chemokines are crucial signaling molecules orchestrating immune responses. Interleukin-8 (IL-8 or CXCL8), a potent CXC chemokine, is primarily responsible for the recruitment and activation of neutrophils to sites of infection or inflammation (Salem, 2022; Mehrabi *et al*., 2019; Ramachandran *et al*., 2018; Matejová *et al*., 2015). In Teleosts, including rainbow trout (*Oncorhynchus mykiss*), IL-8 homologs have been identified and characterized, playing a conserved role in neutrophil chemotaxis and inflammatory responses (Mehrabi *et al*., 2019). Measuring IL-8 gene expression provides a valuable indicator of inflammatory status and immune activation.

The kidney of teleost fish, particularly the anterior kidney (head kidney), is a major lymphoid organ, analogous to mammalian bone marrow, serving as the primary site for hematopoiesis and a key center for immune cell activity, including phagocytosis and cytokine production. Therefore, it is a highly relevant tissue e for assessing systemic immunomodulatory effects.

This study aimed to investigate the effects of dietary supplementation with *Vitex agnus-castus* seed extract (VASE) on the expression of the IL-8 gene in the kidney of rainbow trout. We hypothesized that VASE, through its bioactive components, would modulate IL-8 expression, reflecting potential immunomodulatory activity. The study employed quantitative real-time PCR (qPCR) to measure IL-8 mRNA levels after feeding fish diets containing different concentrations of VASE for 8 weeks.

2. Materials and Methods

2.1. Preparation of *Vitex agnus-castus* L. Seed Extract (VASE)

Commercially sourced dried seeds of *Vitex agnus-castus* L. (certified botanical origin) were pulverized using a laboratory-grade grinder to achieve a homogeneous fine powder.

Sequential extraction was performed employing 70% aqueous ethanol (v/v) as solvent, with exhaustive extraction cycles conducted over 8 h. The resultant crude extract was vacuum-filtered (Whatman No. 1) to remove particulate matter, then concentrated under reduced pressure (Rotary Evaporator) at 40°C. The concentrate was hermetically sealed and stored at -20°C until incorporation into experimental diets.

2.2. Diet Formulation and Proximate Analysis

A nutritionally complete commercial basal diet (45.2% crude protein, 17.8% crude lipid) served as the control formulation. VASE was homogeneously incorporated into the basal diet at three inclusion levels: 0.5% (VASE0.5), 1.0% (VASE1.0), and 2.0% (VASE2.0) (w/w). Mixtures were cold-extruded (≤40°C) to prevent thermal degradation of bioactive compounds, air-dried at 25°C to ≤10% moisture, and stored in vacuum-sealed bags at 4°C.

2.3. Experimental Animals and Husbandry

Juvenile rainbow trout (*Oncorhynchus mykiss*; n=240; mean mass 50.3±5.1 g) were procured from a certified disease-free hatchery. Fish underwent 14-day acclimatization in a recirculating aquaculture system (RAS) under controlled conditions: temperature 15.0±1.0°C, photoperiod 12L:12D, dissolved oxygen 6.8±0.3 mg/L, pH 7.2±0.2, and total ammonia nitrogen <0.1 mg/L. Post-acclimation, fish were randomly distributed into 12 fiberglass tanks (300 L; 20 fish/tank; 3 replicates/treatment). Dietary groups comprised:

- Control: Basal diet
- VASE0.5: Basal + 0.5% VASE
- VASE1.0: Basal + 1.0% VASE
- VASE2.0: Basal + 2.0% VASE

Fish were fed to asymptomatic satiation twice daily (08:00 and 16:00) for 56 days.

2.4. Tissue Sampling and Preservation

Following the 8-week feeding trial, three fish per tank (n=9/treatment) were anesthetized with buffered MS-222 (100 mg/L; Sigma-Aldrich). Kidneys were aseptically dissected, immediately immersed in RNAlater® Stabilization Solution (Thermo-Fisher Scientific), incubated at 4°C for 24 h, then transferred to -80°C for long-term storage .

2.5. RNA Isolation and cDNA Synthesis

Total RNA was extracted from ≈30 mg kidney tissue using TRIzol™ Reagent (Invitrogen™) according to manufacturer specifications. RNA integrity was verified via 1.5% agarose gel electrophoresis (distinct 28S/18S rRNA bands). Concentration and purity were quantified spectrophotometrically (NanoDrop™ 2000; Thermo Fisher) with acceptance criteria: A260/A280 ≥ 1.90, A260/A230 ≥ 2.00. Genomic DNA was eliminated by DNase I treatment (Ambion™). First-strand cDNA was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) with random hexamer primers.

2.6. Quantitative Real-Time PCR (qPCR) Analysis

Primer sequences for target (IL-8; GenBank Accession: EF103443) and reference (β-actin; AF254414) genes were derived from established literature (Table 1). Amplification efficiency (E) was determined from serial dilutions of pooled cDNA (slope= -3.32; R^2>0.99). Reactions (20 µL) contained 10 µL PowerUp™ SYBR™ Green Master Mix (Applied

Biosystems), 0.5 µM each primer, and 2 µL cDNA template (20 ng RNA equivalent). Amplification was performed on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems) under standardized cycling: 95°C for 10 min; 40 cycles of 95°C for 15 sec, 60°C for 60 sec; followed by melt curve analysis (65°C→95°C; +0.5°C/5 sec). Technical triplicates were maintained for all samples.

Table 1. Oligonucleotide primers for qPCR analysis

Gene	Primer Sequence (5' -> 3')	Amplicon Size (bp)	Reference
IL-8	F: CACAGACAGAGAAGGAAGGAAAG R: TGCTCATCTTGGGGTTACAGA	162	(Awad *et al.*, 2011)
β-actin (ACTB)	F: ATGGAAGGTGAAATCGCC R: TGCCAGATCTTCTCCATG	186	(Sigh *et al.*, 2004)

2.7. Statistical Analysis

Relative quantification of IL-8 expression was calculated via; $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001), normalized to the geometric mean of β-actin C_t values. Data are expressed as mean fold-change relative to control ± SEM. Statistical analyses were conducted in SPSS v28.0 (IBM Corp.). Normality (Shapiro-Wilk, $p>0.05$) and homoscedasticity (Levene's test, $p>0.05$) were confirmed by one-way ANOVA. Between-group differences were assessed using Tukey's HSD post-hoc test with significance threshold $\alpha = 0.05$.

3. Results

Dietary supplementation with Vitex agnus-castus seed extract significantly modulated the relative mRNA expression levels of IL-8 in the kidney of rainbow trout in a dose-dependent manner (Figure 1).

Control vs. VASE0.5: Fish fed the diet containing 0.5% VASE showed no significant difference ($p=0.458$) in IL-8 expression compared to the control group (fold change= 1.2±0.3).

Control vs. VASE1.0: Fish fed the 1.0% VASE diet exhibited a significant upregulation ($p<0.01$) of IL-8 gene expression. The mean fold change relative to the control was 2.8±0.4.

Control vs. VASE2.0: Fish fed the highest dose (2.0% VASE) showed a significant downregulation ($p<0.05$) of IL-8 expression. The mean fold change relative to the control was 0.6±0.1.

Comparison between VASE groups: The expression level in the VASE1.0 group was significantly higher than both the VASE0.5 group ($p<0.01$) and the VASE2.0 group ($p<0.001$). The VASE2.0 group expression was significantly lower than both the VASE0.5 ($p<0.05$) and VASE1.0 groups.

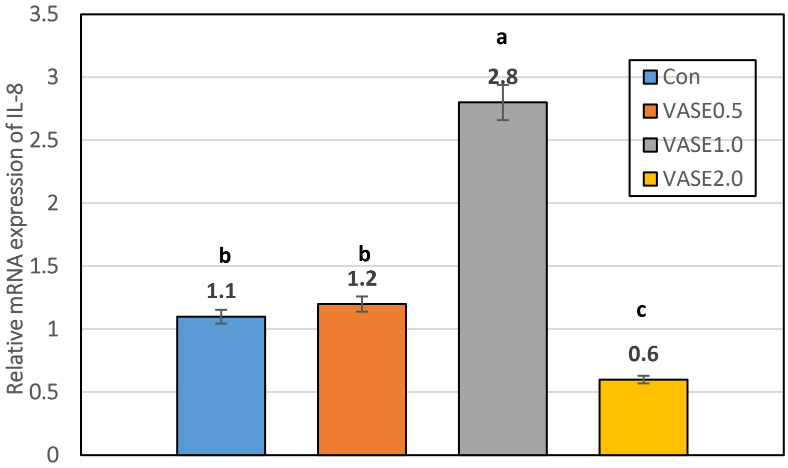

Figure 1. Dose-dependent modulation of renal IL-8 mRNA expression in rainbow trout (*Oncorhynchus mykiss*) fed *Vitex agnus-castus* L. seed extract (VASE) supplemented diets for 8 weeks.

Relative IL-8 transcript levels were quantified by qPCR and normalized to the endogenous reference gene β-actin. Data are expressed as fold-change relative to the control group (assigned a value of 1.0). Vertical bars represent mean values ±SEM (n= 9 biological replicates per treatment group). Distinct lowercase letters (a, b, c) denote statistically significant intergroup differences ($p < 0.05$) as determined by one-way ANOVA with Tukey's HSD post-hoc analysis. Con: Control; VASE0.5: 0.5% extract; VASE1.0: 1.0% extract; VASE2.0: 2.0% extract.

4. Discussion

This study provides the first evidence that dietary supplementation with *Vitex agnus-castus* seed extract (VASE) modulates the expression of the pro-inflammatory chemokine IL-8 in the kidney of rainbow trout, demonstrating dose-dependent immunomodulatory activity.

The significant upregulation of IL-8 mRNA (2.8-fold) observed in the kidney of fish fed the 1.0% VASE diet suggests a stimulatory effect on this key chemokine pathway at this moderate dose. IL-8 is a crucial mediator of acute inflammation, primarily responsible for recruiting neutrophils to sites of infection or tissue damage (Bilen & Elbeshti, 2019). Enhanced expression could indicate a state of heightened immune alertness or readiness. This aligns with studies on other plant-derived immunostimulants (e.g., Echinacea, garlic, mushroom polysaccharides) in fish, which often show upregulated expression of pro-inflammatory cytokines and enhanced phagocytic activity following moderate supplementation (Tafi *et al*., 2018; Haghighi *et al*., 2017). Bioactive compounds in VASE, such as iridoids (aucubin & agnuside) and flavonoids (casticin), possess documented anti-inflammatory and immunomodulatory properties, sometimes exhibiting biphasic effects (stimulatory at low/moderate doses) (Salem & Mohamed, 2025). These compounds may interact with fish immune cells (e.g., macrophages, neutrophils) in the kidney, potentially through pattern recognition receptors or signaling pathways (e.g., NF-κB), leading to increased IL-8 transcription.

Conversely, the significant downregulation of IL-8 expression (0.6-fold) observed at the highest dose (2.0% VASE) points towards a potential anti-inflammatory or immunosuppressive effect at this concentration. This highlights the crucial importance of dosage in phytogenic supplementation. High doses of bioactive plant compounds can sometimes exert inhibitory effects on immune responses (Haghighi *et al*., 2017; Zhou *et al*., 1998). Compounds like casticin have demonstrated potent anti-inflammatory effects in mammals by inhibiting pro-inflammatory cytokine production and NF-κB signaling

(Altunoglu *et al*., 2017). The suppression of IL-8 at 2.0% VASE could reflect an overstimulation leading to feedback inhibition, a direct suppressive effect of high concentrations of specific compounds, or potentially the activation of anti-inflammatory pathways. This finding underscores that "more is not always better" and high doses might even be detrimental to optimal immune function.

The lack of significant effect at the lowest dose (0.5% VASE) suggests that this concentration was insufficient to elicit a measurable immunomodulatory response on IL-8 expression under the conditions of this study.

The kidney, being a major immune organ in fish, is an appropriate site to detect systemic immunomodulation. Changes in IL-8 expression here likely reflect alterations in the baseline inflammatory tone or the responsiveness of immune cells within this critical tissue. It is important to note that gene expression changes do not directly equate to functional outcomes (e.g., neutrophil migration, disease resistance). Future studies should correlate IL-8 expression with functional immune assays (e.g., phagocytosis, respiratory burst, lysozyme activity) and, crucially, with challenge tests against relevant pathogens (e.g., *Aeromonas salmonicida, Yersinia ruckeri*) to determine the biological significance of the observed modulation. The moderate upregulation seen at 1.0% might enhance resistance to bacterial infections where neutrophil recruitment is vital, while the downregulation at 2.0% might compromise defense or potentially protect against chronic inflammation.

5. Conclusion

This study demonstrates that dietary supplementation with *Vitex agnus-castus* seed extract significantly modulates the expression of the IL-8 gene in the kidney of rainbow trout in a dose-dependent manner. A moderate inclusion level (1.0%) stimulated IL-8 expression, potentially indicating enhanced immune readiness, while a high inclusion level (2.0%) suppressed it, suggesting anti-inflammatory or immunosuppressive effects. The 0.5% inclusion had no significant effect. These findings reveal the immunomodulatory potential of VASE in an important aquaculture species. The dose-dependent biphasic effect underscores the critical need for optimizing supplementation levels. Further research is warranted to investigate the effects of VASE on other immune genes and functional immune parameters; determine the impact of VASE supplementation on disease resistance against bacterial and viral pathogens; identify the specific bioactive compounds in VASE responsible for the observed immunomodulation; explore longer-term effects and optimal feeding durations; and assess potential effects on reproduction or stress tolerance, given Vitex's known hormonal activities in mammals. VASE shows promise as a natural immunomodulator in rainbow trout aquaculture, but careful dosage selection is paramount to achieve the desired beneficial effects without immune suppression.

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