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Development of Lupeol fortified Fish Meal to Reduce Oxidative Stress

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KEYWORDS

Sustainable aquaculture; Lupeol; Fish meal; Oxidative stability; Lipid peroxidation; Antioxidant

ABSTRACT:

Introduction: Lupeol is a naturally occurring pentacyclic triterpenoid found in fruits and medicinal plants such as mangoes, olives, and aloe vera. It possesses strong antioxidant properties that help suppress inflammation and prevent diseases like cardiovascular conditions and diabetes. Lupeol also demonstrates anticancer effects by inhibiting the growth of cancer cells and promoting apoptosis without harming normal cells. It enhances the body's antioxidant defense by stimulating enzymes like superoxide dismutase and catalase, reducing oxidative stress linked to chronic illnesses and aging. In aquaculture, lupeol helps protect fish from oxidation, improving their health, shelf life, and the nutritional quality of fish-based food products.

Objectives: This study explores the antioxidant and free radical-scavenging properties of lupeol, aiming to enhance fish health, nutrition, and market value through its incorporation into fish feed.

Methods: Neem bark, rich in lupeol, was collected, sun-dried, powdered, and stored. Lupeol was extracted using hexane due to its non-polarity and high solvating power. A 1:10 ratio of bark to hexane was used. Cell disruption was achieved using a 42 kHz ultrasonic sonicator for 30 minutes. The mixture was filtered and distilled to remove hexane. TLC confirmed presence of lupeol, while UV-Vis spectrophotometry quantified it at 200 nm. DPPH assay tested antioxidant activity before and after lupeol was added to commercial and formulated fish feed. Tests like MDA, ammonia, and nitrate assessed oxidative stress and fish health improvements.

Results: The study demonstrated lupeol's strong antioxidant properties through the DPPH assay, which showed increased activity with higher concentrations. A slight decrease in activity was noted when lupeol was added to commercial fish feed, likely due to ascorbic acid interference, which was mitigated by using homemade fish feed. Ammonia and nitrate tests showed reduced levels in lupeol-fed fish, indicating lower oxidative stress. Microbial analysis revealed fewer harmful bacteria, suggesting improved gut health. The MDA assay confirmed reduced lipid peroxidation and oxidative damage. Overall, lupeol effectively reduced oxidative stress and enhanced fish health, highlighting its potential as a natural antioxidant in aquaculture.

Conclusions: Lupeol in fish feed reduces oxidative stress, enhances immunity, boosts metabolism, and improves fish vitality. As a natural antioxidant, it offers a sustainable alternative to chemical additives. The study shows improved water quality and gut health, but further research is needed to optimize dosage and evaluate long-term, large-scale use.

1. Introduction

Lupeol is a naturally occurring pentacyclic triterpenoid compound, abundantly present in various fruits, vegetables, and medicinal plants. Common dietary and medicinal sources include mangoes, olives, grapes, strawberries, aloe vera, dandelion, and white mulberry [1]. Structurally, lupeol is a lupane derivative with a characteristic five-ring framework, a hallmark of triterpenoids [2]. Owing to its wide distribution in natural sources, lupeol has garnered significant attention for its pharmacological and therapeutic properties, notably its antioxidant and anticancer activities.

One of lupeol's most profound features is its strong antioxidant property, which plays a vital role in minimizing inflammation and oxidative stress, thereby reducing complications associated with cardiovascular diseases, diabetes, and neurodegenerative disorders [3,7].

Journal of Chemical Health Risks

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In the context of cancer therapy, lupeol has been reported to suppress the proliferation of prostate, breast, and pancreatic cancer cells by modulating key signaling pathways such as PI3K/Akt and NF-κB, and by inducing apoptosis selectively in malignant cells without harming normal tissues [4–6].

Lupeol's antioxidant activity functions on two fronts: (i) direct scavenging of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and (ii) indirect enhancement of endogenous antioxidant enzymes like superoxide dismutase (SOD), catalase, and glutathione peroxidase [8]. These enzymes contribute significantly to neutralizing free radicals and preventing lipid peroxidation, a major process underlying oxidative cell damage.

Fish, particularly oily species rich in polyunsaturated fatty acids (PUFAs), are highly susceptible to oxidative spoilage. Lipid oxidation occurs when unsaturated fatty acids react with oxygen, producing hydroperoxides, aldehydes, and other reactive compounds [9–10]. Factors like exposure to air, light, and improper storage exacerbate this process, leading to rancidity, off-odours, discoloration, and nutritional degradation—especially the breakdown of beneficial omega-3 fatty acids such as EPA and DHA [11–13].

Typically, antioxidants such as vitamin E and synthetic compounds are added to fish feeds or packaging systems to prevent oxidation. However, there is growing interest in replacing synthetic additives with natural bioactives. Lupeol, due to its strong free radical scavenging ability, presents a sustainable alternative [14–15].

In fish, lupeol inhibits lipid peroxidation by neutralizing free radicals and upregulating enzymatic antioxidants. This not only protects fish tissues from degradation but also maintains sensory quality, nutritional value, and shelf life. Moreover, it suppresses the formation of malondialdehyde (MDA) and other toxic secondary oxidation products [16–17].

2. Objectives

The primary objectives of the present study are:

To begin with to investigate the antioxidant activity of lupeol, with a specific focus on its oxidation-inhibition potential and free radical-scavenging properties.

Further, to evaluate the efficacy of lupeol as a natural antioxidant additive in fish feed for: Enhancing the health and physiological development of the fish, preserving the nutritional quality of fish meal, and delivering health benefits to consumers through improved fish-based products.

Finally, to assess the impact of lupeol supplementation on improving the growth performance of the target aquatic species and its contribution to the economic and market value of the final product.

3. Methods

3.1. Materials

Many plants are known to contain lupeol; however, we selected neem bark for its isolation because it contains a relatively higher concentration of lupeol compared to other botanical sources[18]. The neem bark, derived from the neem tree (*Azadirachta indica*), was collected from a specific region in Coimbatore. To reduce its moisture content—an essential step in preventing microbial growth and enhancing the efficiency of subsequent extraction processes—the collected bark was sun-dried for two days. This drying step plays a crucial role in preserving the stability of bioactive compounds and protecting them from degradation caused by moisture or microbial activity.

Following the drying process, the bark was ground into a fine powder using a mixer, effectively increasing the surface area for improved solvent extraction. The powdered neem bark was then stored in an airtight container to prevent contamination and degradation due to air and moisture exposure. As a triterpene, lupeol is poorly soluble in aqueous solutions but dissolves readily in organic solvents such as ethane, ethanol, and hexane[19]. Among these, hexane was chosen as the extraction solvent based on supporting literature, due to its high solvating ability for lupeol and its non-polar nature, which makes it particularly suitable for extracting hydrophobic compounds. Additionally, hexane's relatively low boiling point facilitates its evaporation during the concentration phase and allows for its easy removal from the extract.

A total of 200 grams of ground neem bark was mixed with 2000 millilitres of hexane, maintaining a 1:10 ratio. This ratio was adopted to ensure effective extraction of lupeol and other non-polar constituents from the plant

Journal of Chemical Health Risks
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material. The mixture was allowed to stand undisturbed for a specific period, enabling the diffusion of lupeol into the hexane solvent. Solvent extraction operates on the principle that compounds with similar polarities dissolve one another; hence, non-polar compounds like lupeol are efficiently solubilized in non-polar solvents such as hexane.

3.2. Cell Disruption

To efficiently extract lupeol from the plant cell matrix, a 42 kHz ultrasonic sonicator was employed. This high-frequency ultrasonic process induces the formation of cavitation bubbles, which implode to generate localized zones of high pressure and temperature. These implosions disrupt the plant cell walls, thereby facilitating the release of lupeol into the hexane solvent.

The sonication was carried out for 30 minutes, comprising 5-minute sonication cycles followed by 1-minute resting intervals to prevent thermal degradation of the heat-sensitive bioactive compounds. This approach enhances the extraction process while preserving the integrity of lupeol and other active constituents[20]. The use of ultrasonic energy significantly improves extraction efficiency and simultaneously maintains the structural integrity of the sample for subsequent purification and analysis.

Following the sonication, the mixture was filtered using Whatman filter paper to remove insoluble plant residues. Post-filtration, a yellowish clear liquid was obtained, free from any undissolved particulates.

3.3. Distillation

To concentrate the sample and remove excess hexane, simple distillation was employed. This method utilizes the difference in boiling points, with hexane boiling at approximately 69°C. The filtered extract was placed in a round-bottom flask, which was connected to a distillation apparatus comprising a condenser and a receiving flask. The apparatus was gradually heated to 70°C, causing the hexane to evaporate. The vapor then passed through the condenser, where it was cooled and condensed back into a liquid. The condensed hexane was collected in the receiving flask, leaving behind a concentrated extract with a high lupeol content in the original flask, ready for storage and further analysis.

3.4. Qualitative and Quantitative Analysis

For qualitative analysis, Thin Layer Chromatography (TLC) was performed to confirm the presence of lupeol post-distillation[21]. Silica gel was used as the stationary phase, with hexane and ethyl acetate in an 8:2 ratio as the mobile phase. A small amount of the lupeol extract was applied to the TLC plate. A yellow band indicated the presence of phytochemicals, while iodine vapor was used to visualize the otherwise colorless lupeol under UV light. Three bands were visible, indicating stable phytochemicals. The stability of lupeol at the distillation temperature of 70°C is demonstrated by its high boiling point of 212°C, ensuring its integrity at temperatures below 200°C.

Quantitative analysis of lupeol was conducted using UV-Vis spectrophotometry[22]. Since lupeol is colorless, the analysis was carried out at a wavelength of 200 nm, the optimal wavelength for lupeol's absorption. The sample was scanned across the ultraviolet spectrum, and the results revealed sharp peaks corresponding to lupeol and other phytochemicals present in the neem bark extract. The peak intensities provided information on the concentration of lupeol, enabling accurate quantification and further evaluation of the compound's purity and stability.

3.5. Analysis and Observation

The DPPH assay was performed to evaluate the antioxidant activity of lupeol before and after its incorporation into fish feed. The DPPH assay is a simple, cost-effective method for assessing a compound’s ability to scavenge free radicals, widely used in research to screen and quantify antioxidant activity in various substances.

The antioxidant activity was measured using a spectrophotometer. The extracted lupeol was incorporated into both commercial and formulated fish feeds to assess its antioxidant capacity in the fish feed context. Fish were fed with either commercial fish feed (control group) or lupeol-fortified fish feed (treatment group). The activity of both groups was monitored to determine whether the incorporation of lupeol resulted in a reduction of oxidative stress.

To evaluate the reduction in oxidative stress, several tests were performed on fish fed the lupeol-fortified feed. These included ammonia and nitrate tests, microbial

Journal of Chemical Health Risks
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analysis, and the Malondialdehyde (MDA) biomarker assay, which collectively assess oxidative damage and metabolic efficiency. Furthermore, the weight and swimming speed of the fish were measured before and after the lupeol incorporation to analyze the impact of lupeol on fish health and overall activity levels.

4. Results and Discussion

4.1. DPPH Assay Before adding Lupeol

Figure 1 shows the antioxidant activity of lupeol before adding it to fish feed. DPPH assay is a valid method for the determination of antioxidant capacity of the sample[23]. Varied concentrations of lupeol (800 µg, 400 µg, 200 µg, 100 µg, 50 µg, and 25 µg) were used because lupeol is water-insoluble; therefore, ethanol was used to dissolve the lupeol.

1 ml of DPPH solution was added to 2 ml of the sample aliquot and the sample was incubated in the dark for about 30 minutes. After incubation, the absorbance was taken spectrometrically at 517 nm using a spectrophotometer, and the results were calculated by using the following formula,

$$\text{Antioxidant Activity}(\%) = \frac{\text{AbsControl} - \text{AbsSample}}{\text{AbsControl}} \times 100 \quad (1)$$

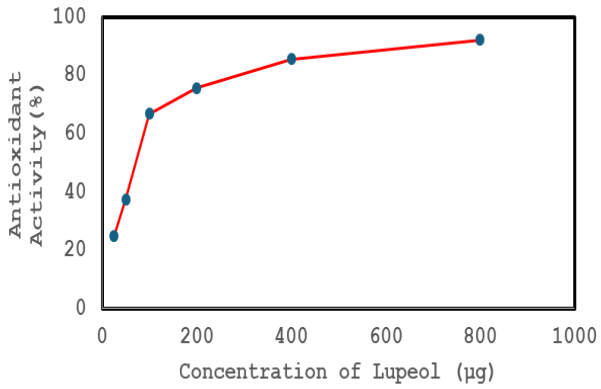

Figure 1. Antioxidant activity of Lupeol -Pre incorporation.

The results indicated that the antioxidant activity of lupeol was concentration dependent. The highest antioxidant activity (92.15%) was observed at 800 µg, while the lowest (24.98%) was at 25 µg. This demonstrates that increasing the concentration of lupeol enhances its ability to neutralize free radicals, confirming its potent antioxidant properties.

4.2. DPPH Assay After adding Lupeol

Figure 2 provides the antioxidant activity of lupeol after fortification with commercial fish feed. DPPH Assay was performed for the lupeol sample after adding fish feed to it and the absorbance value was obtained spectrometrically.

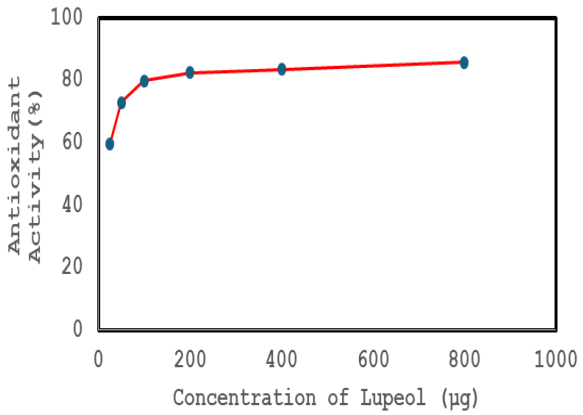

Figure 2. Antioxidant Activity of Lupeol-Post incorporation with commercial fish feed

The commercial fish feed was ground into a fine powder using a mortar and pestle. Lupeol in powder form (100 µg) was mixed with a small amount of ethanol to enhance binding and was thoroughly blended with the fish feed. The lupeol fortified fish feed was subjected to DPPH assay to evaluate its antioxidant activity.

The results showed that the antioxidant activity at 25 µg was 59.79 percentage. By comparing table 1 and table 2, it shows increased antioxidant activity in lower concentrations. However, in higher concentration such as 800 µg, the antioxidant activity of lupeol decreased from 92.15% to 85.77%. The reason behind the reduction of antioxidant activity is ascorbic acid, which was presented in the commercial fish feed. This reduction was attributed to the presence of ascorbic acid in commercial fish feed. This antioxidant substance suppressed the activity of the lupeol.

Thus, DPPH assay showed decreased antioxidant activity. To resolve this issue, a homemade fish feed was formulated using carrot, wheat and soybean and incorporated with lupeol. The homemade fish feed

Journal of Chemical Health Risks
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fortified with lupeol was subjected to DPPH assay, and the results demonstrated improved antioxidant activity, overcoming the interference caused by ascorbic acid in commercial feed.

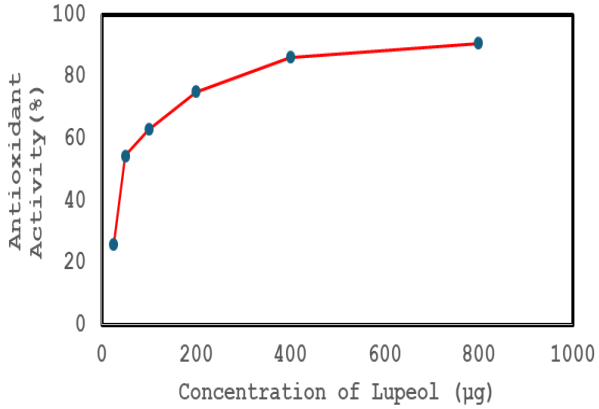

Figure 3. Antioxidant Activity of Lupeol-Post incorporation with formulated fish feed

Figure 3 shows the results of DPPH assay for fish feed which is formulated and incorporated with lupeol. From the tabular column, it is identified that the antioxidant activity increased at higher concentrations. This test ensures that lupeol is a potential component which contains effective antioxidant property.

Thus, this result confirmed the antioxidant property of lupeol which will effectively reduce the oxidative stress produced inside the aquatic animals.

4.3. Effect of Ammonia Test

Ammonia is a vital indicator of aquatic fish health. It is a byproduct of protein metabolism and is excreted primarily through fish gills. Elevated ammonia is highly toxic and causes excessive stress, injury to the gills, and respiratory distress in fish[24]. Toxicity of ammonia is pH dependent, but in low pH, ammonia exists in the ammonium ion (NH_4^+) form, which is less toxic. Elevated ammonia inhibits cellular function and causes oxidative stress by forming reactive oxygen species (ROS), which damage proteins, lipids, and DNA. Oxidative stress weakens the immune system, slows growth, and renders fish more susceptible to infection and disease.

Accordingly, ammonia test was conducted, in order to check the concentration of ammonia in fish pot water, 5 ml water from both the control group fed with commercial fish feed and the treatment group fed with lupeol enriched fish feed was taken in different test tubes. Two drops of phenolphthalein indicator solution were placed in each of the samples. The test tubes were left undisturbed for 20 minutes to allow the reaction to develop. The intensity of the pink colour of the samples indicated the concentration of ammonia, with the deeper pink colour corresponding to higher concentration of ammonia.

a **b**

Figure 4. Ammonia test results: a. control group, b. treatment group

Figure 4 reveals that the control group exhibited high ammonia levels due to oxidative stress, which occurs when reactive oxygen species (ROS) overwhelm the fish's antioxidant defences, causing cellular damage. Conversely, the treatment group showed lower ammonia levels, indicating reduced oxidative stress. This reduction is attributed to lupeol, a natural antioxidant that neutralizes ROS and protects cellular functions. Consequently, the fish in the treatment group experienced improved ammonia detoxification and better health, demonstrating lupeol's effectiveness in mitigating oxidative stress in aquatic environments.

4.4. Effect of Nitrate Test

Nitrate (NO_3^-), the end product of the nitrogen cycle, can build up in aquatic systems and induce oxidative stress in fish populations[25]. Hence, the concentration of nitrate can be utilized as an indicator of the severity of oxidative stress. To determine this, a nitrate assay was conducted. For this, 5 mL water samples of the control and treatment groups were taken in separate test tubes. Each of these samples was treated with 1 mL cadmium

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JCHR (2025) 15(3), 2384-2392 | ISSN:2251-6727

acetate reagent and incubated for 10-15 minutes. After incubation, 0.1 mL of sulphanilamide solution was added and left to react for 2-3 minutes. This was followed by the addition of 0.1 mL of NED solution, followed by leaving the mixture to react for 10-15 minutes. The concentration of nitrate was determined calorimetrically at 540 nm wavelength.

Calorimetric readings gave a reading of 0.526 for the control and 0.26 for the treatment group. The concentration of nitrate was calculated based on the formula,

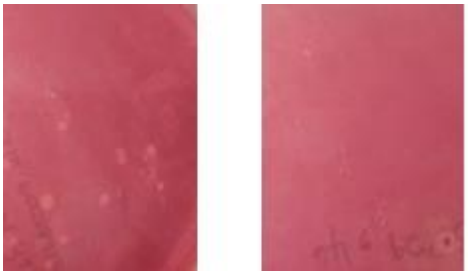
$$C = \frac{A}{\varepsilon \times l} \qquad (2)$$

The calorimetric values were recorded at 0.526 in the experimental group and 0.26 in the comparison group. The nitrate concentrations accompanying these values were found to be 4.208 ppm for the comparison group and 2.08 ppm for the experimental group. The results represent a significant reduction of nitrate levels in the experimental group compared to the comparison group, suggesting that the intervention effectively reduced nitrate accumulation, and thus the production of oxidative stress in the experimental group.

4.5. Microbial Analysis

Microbial analysis is a useful method for quantifying oxidative stress in fish. Oxidative stress weakens the immune system, which predisposes fish to toxic Gram-negative bacteria like E. coli and Salmonella that inhabit the gastrointestinal tract. Microorganisms that the immune system is unable to eliminate can be isolated from fish feces. The quantity of such microorganisms is an indication of the level of oxidative stress. This method offers a straightforward means of quantifying the impact of oxidative stress on fish health and the antioxidant activity of lupeol in enhancing immune function. Faecal samples from fish were collected from both the control and treatment groups. Using the spread plate method, these samples were then plated on MacConkey Agar, a selective medium for the growth of bacteria. The plates were incubated at 37°C for 24 hours. This method allows for the identification and measurement of Gram-negative bacteria, which are often associated with gut health and can indicate levels of oxidative stress[26].

A comparison of bacterial growth patterns between the control and treatment groups, as shown in Figure 5, shows that the reduction in bacterial presence in the treatment group that was given lupeol-enriched fish feed indicates improved gut health and reduced oxidative stress.



a **b**

Figure 5. Microbial analysis results: a. control group, b. treatment group

4.6. MDA Biomarker Assay

The Malondialdehyde (MDA) Biomarker Assay is one common technique for determining oxidative stress through the measurement of lipid peroxidation, where reactive oxygen species (ROS) target cell membrane lipids, resulting in the production of MDA as a by product[27]. High levels of MDA are an indicator of cellular injury and oxidative stress, which are commonly associated with different diseases and environmental stressors in fish. Through the comparison of MDA content among various experimental groups, determine the degree of oxidative damage and evaluate the efficacy of possible treatments in improving the general health and well-being of aquatic organisms.

The method starts with the accumulation and homogenization of fish tissue or blood sample in a buffer solution to release cellular components. The sample is combined with thiobarbituric acid (TBA) reagent, which interacts with MDA in acidic conditions at high temperature (90-100°C) to give a pink MDA-TBA adduct. The mixture is incubated at 60-90 minutes and centrifuged to eliminate debris. The MDA-TBA complex is read at 532 nm by a spectrophotometer[28]. MDA concentration is estimated using formula (2).

The results from the MDA Biomarker Assay reveal a significant difference in MDA concentrations between the control and treatment groups. The control group exhibited an MDA concentration of 2.09 μM, indicating higher levels of lipid peroxidation and oxidative stress.

Journal of Chemical Health Risks

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JCHR (2025) 15(3), 2384-2392 | ISSN:2251-6727

In contrast, the treatment group showed a reduced MDA concentration of 0.66 µM, suggesting a lower level of oxidative damage. This reduction in MDA levels demonstrates the effectiveness of the treatment in mitigating oxidative stress, thereby protecting the cellular integrity of the fish and promoting overall health.

5. Conclusion

This research fully proves the antioxidant efficacy of lupeol when added to fish feed and its application in reducing oxidative stress in aquatic organisms. Conclusions drawn from the research were proven using various analysis techniques involving the DPPH assay, the MDA biomarker assay, nitrate and ammonia determination, and microbial screening. The DPPH assay indicated the typical antioxidant activity of lupeol, which was slightly decreased upon blending with commercial fish feed because of the presence of ascorbic acid. This deficiency, however, was overcome through the use of homemade fish feed preparation, where lupeol was able to reveal its complete antioxidant efficacy.

The MDA biomarker assay confirmed a notable decrease in lipid peroxidation, indicating a decrease in oxidative stress in the treatment group. The nitrate and ammonia tests further indicated increased efficiency in metabolism and an improved aquatic environment. Microbial screening, as conducted on MacConkey Agar indicated minimal bacterial growth in the fish feces when fed with lupeol enriched feed, indicating improved immune function and gut health. The significance of lupeol supplementation in aquaculture nutrition is its ability to counteract oxidative stress, a major problem in the industry. Oxidative stress is a condition caused by an imbalance between reactive oxygen species (ROS) and the defense system of the organism, leading to cellular damage and impaired fish health.

Lupeol, a triterpenoid compound found naturally, is able to neutralize free radicals and thereby impart protection against fish cell damage caused by oxidative stress. Its protective action translates into improved immune functions, improved digestion, and overall improved vitality. Lupeol is also a green, natural alternative to chemical antioxidants, thereby ensuring sustainable aquaculture management while reducing the risk of chemical residue contamination in fish products.

The reduction of nitrate and ammonia content in the treatment group also suggests an improvement in metabolic efficiency and water quality, both of which are important parameters for maximum fish growth and productivity. The major benefits of lupeol enrichment in aquaculture diets include reduced oxidative stress, enhanced immune responses, enhanced metabolic efficiency, increased activity and vigor of fish, and the facilitation of sustainable and eco-friendly aquaculture operations. All these benefits collectively lead to enhanced fish productivity and better health outcomes. However, more studies are needed to study the long-term effects of lupeol on fish growth, reproduction, and immune responses, and its effectiveness on different fish species. It is also important to identify the most effective dosage of lupeol that ensures maximum antioxidant activity without inducing any adverse side effects.

Future studies should also study the ecological effect of lupeol on water quality and determine economically feasible means of large-scale production to further enhance its practical application in the aquaculture industry. In conclusion, lupeol enrichment in fish feed effectively reduces oxidative stress, improves immune response, enhances metabolic efficiency, and improves overall fish health. The significant reduction in MDA levels, lowered nitrate and ammonia levels, and minimal or no microbial growth in the treatment group validate the antioxidant activity of lupeol. These findings validate the potential of lupeol as a natural antioxidant for sustainable aquaculture, opening the door to improved fish productivity and reduced environmental impact. With further research and large-scale implementation, lupeol can revolutionize the aquaculture industry, providing healthier and more sustainable fish feed products.

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