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Exploring the Potential of Mangrove Leaf Extracts as Natural Preservatives for Protein-Rich Fish: Evidence from *Chanos chanos*

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ABSTRACT

Purpose of the study: This study aims to analyze the effect of ethanolic extract of *Rhizophora mucronata* leaves at different concentrations and preservation times on the protein content of milkfish (*Chanos chanos*), to determine the optimal extract concentration for maintaining protein quality during storage.

Methodology: This study used an experimental laboratory design with milkfish (*Chanos chanos*) treated using ethanolic extracts of *Rhizophora mucronata* leaves. Tools included a rotary evaporator (IKA RV 10), analytical balance (Ohaus Pioneer), freeze dryer (Labconco), and Kjeldahl apparatus (Velp Scientifica). Protein analysis followed AOAC (2005) using SPSS v26 for Two-Way ANOVA.

Main Findings: The study found that increasing concentrations of ethanolic extract of *Rhizophora mucronata* leaves led to higher protein content in milkfish (*Chanos chanos*). The highest protein level was observed at 50 ppm concentration with 12-hour preservation. Two-Way ANOVA showed significant effects of concentration and time individually, but no significant interaction between the two factors ($P = 0.148 > 0.05$).

Novelty/Originality of this study: This study is the first to evaluate the effect of *Rhizophora mucronata* ethanolic leaf extract on the protein content of milkfish (*Chanos chanos*) under Philippine conditions. It introduces a natural, eco-friendly preservation approach, advancing current knowledge on the use of mangrove bioactive compounds to maintain fish protein quality during post-harvest storage.

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1. INTRODUCTION

Milkfish (*Chanos chanos*), known locally as *bangus* in the Philippines, is one of the country's primary aquaculture commodities and contributes a substantial share of animal protein supply for coastal households as well as the general population. This role makes it crucial to national food security [1]. Nutritional survey data and national reports indicate that fishery products, including milkfish, account for a significant portion of the Filipino population's animal protein intake, with fish and seafood contributing approximately 42.2% according to FNRI (FINS report), and other estimates suggesting that fish contribute up to around 50% of total animal protein, depending on the calculation method [2]. In addition to quantity, the nutritional content of milkfish is

relatively high including quality protein, unsaturated fatty acids (omega-3) and several important micronutrients thus contributing to the nutritional status of pregnant women and child growth in coastal communities; This nutritional composition has been documented in studies of amino acid and fat composition in Chanos chanos. [3]. Economically, milkfish aquaculture provides livelihoods for fish farmers, processors, and actors along the local to export value chain, and it remains one of the pillars of national aquaculture production, continually supported by fisheries sector policies [4], [5]. Given its nutritional and economic significance, maintaining protein quality and post-harvest product quality has become a priority in the Philippines' food security and safety policies, including efforts to reduce post-harvest losses and adopt safe and sustainable preservation techniques.

Milkfish protein quality can deteriorate after harvest due to oxidation processes, proteolytic enzyme activity, and suboptimal storage conditions, leading to denaturation and loss of nutritional value. Studies indicate that fish protein can degrade during extended frozen storage or significant temperature fluctuations, resulting in formaldehyde formation and changes in texture, taste, and odor [6]-[8]. While chemical preservatives are often effective, they raise concerns regarding chemical residues and consumer preference for natural, safe, and environmentally friendly substances [9]-[11]. Therefore, natural solutions as protein preservatives and antioxidants have begun to attract attention in fisheries research [12]-[14]. Exploration of local plant extracts with antioxidant and antimicrobial activity is expected to help maintain fish protein levels post-harvest and throughout the supply chain [15], [16].

Mangrove leaves from *Rhizophora mucronata* contain bioactive compounds such as flavonoids, tannins, and phenolics, which exhibit strong antioxidant activity and the ability to neutralize free radicals as well as prevent lipid and protein oxidation. Several chemical studies have reported significant antioxidant activity of *R. mucronata* stem and leaf extracts using DPPH and total phenolic assays [17], [18]. Other investigations of these mangrove leaves have shown antibacterial activity against pathogens such as *Ralstonia solanacearum* and *Helicobacter pylori*, indicating their potential as natural preservatives for fish- or animal-based products [19], [20]. The Philippines has extensive *R. mucronata* mangrove areas and ecosystems that support mangrove growth, providing a locally and sustainably available source of ethanol-extracted mangrove leaves [21], [22]. Thus, the local use of mangrove leaves as a functional material offers a practical intervention for preserving milkfish protein quality in the Philippines.

Previous studies have evaluated various aspects of *Rhizophora mucronata* extracts, including antioxidant activity of stems and leaves, antimicrobial effects, and the formulation of tablets from black mangrove fruit with DPPH testing to determine antioxidant IC50. Other research has tested the efficacy of ethanol leaf extracts in wound healing in experimental animals infected with *Staphylococcus aureus* [23]. However, few studies have specifically evaluated changes in fish protein content after treatment with *R. mucronata* ethanol extracts, particularly in the local Philippine context. No studies have systematically compared different concentrations of mangrove leaf extract on milkfish protein content during storage or post-harvest treatments in the Philippines. Therefore, this research addresses a knowledge gap regarding the effect of mangrove leaf extracts on milkfish protein quality in the local context.

The urgency of this study arises from the need to enhance the quality and nutritional value of milkfish, which is critical for food security and the economy of coastal communities in the Philippines, while meeting consumer demand for high-quality fish products. The main objective of this research is to analyze changes in milkfish protein content following the addition of *Rhizophora mucronata* leaf ethanol extract at various concentrations and storage intervals. The study also aims to determine the optimal extract concentration that is most effective in maintaining protein levels and preventing nutritional degradation. Additionally, it will examine whether the treatment affects other quality parameters such as odor, texture, and color, which are often indicators of protein spoilage. The findings are expected to provide empirical data that can be translated into practical applications for the fisheries industry and small-scale enterprises in the Philippines.

Scientifically, this research will expand understanding of the mechanisms by which antioxidant compounds in *R. mucronata* leaf extracts protect fish protein structure from oxidation and denaturation, including at the biochemical and molecular levels. Practically, the results are expected to present a natural preservative option that is affordable and accessible to fish farmers and small-to-medium enterprises in the Philippines, thereby increasing the added value of milkfish products and reducing post-harvest losses. Environmental benefits are also anticipated, as the use of local natural materials such as mangrove leaves reduces reliance on synthetic chemicals with potential pollution risks, while promoting mangrove conservation as a vital biological resource. This study also enables integration between mangrove ecology and fish production, supporting sustainable coastal development. Ultimately, the results could provide a basis for fisheries policy recommendations, seafood processing practices, and milkfish product quality standards in the Philippines.

2. RESEARCH METHOD

2.1. Research Design

This study was an applied laboratory experiment aimed at analyzing the effect of ethanol extract of mangrove leaves (*Rhizophora mucronata* Lamk.) on the protein content of milkfish (*Chanos chanos*). The research employed a factorial experimental design (Two-Way ANOVA) with two treatment factors, namely:

- Factor A: Concentration of *R. mucronata* leaf ethanol extract (12.5, 25, 37.5, 50 ppm, and a control without extract)
- Factor B: Soaking duration (12, 24, 36, and 48 hours).

Each treatment combination was conducted in duplicate. This design allowed evaluation of the main effects of concentration and soaking time, as well as their interaction, on the changes in milkfish protein content.

2.2. Materials

Biological material: Fresh milkfish with an average weight of ±100 g and length of ±20 cm. Chemical reagents: 96% food-grade ethanol, concentrated H_2SO_4, 50% NaOH, selenium powder, 4% boric acid, phenolphthalein (PP) and methyl red (MR) indicators, 0.02 N HCl, and distilled water. Main equipment: Blender, rotary evaporator, freeze dryer, analytical balance, Kjeldahl digestion–distillation–titration apparatus, and glass jars for soaking treatments.

2.3. Preparation of Mangrove Leaf Ethanolic Extract

A total of 4.5 kg of *Rhizophora mucronata* leaves were air-dried at room temperature until reaching a low moisture content, then blended into powder. The drying process of mangrove leaves is illustrated in Figure 1.

Figure 1. Drying process of *Rhizophora mucronata* leaves prior to extraction

The powdered leaves were macerated with 2 L of 96 % ethanol for 2 × 24 h. The filtrate was evaporated using a rotary evaporator at 60 °C for 4 h to obtain a viscous extract, which was further dried with a freeze dryer for 3 h to form a gel. The stock solution was prepared by dissolving the gel extract in a small amount of ethanol and diluting it with distilled water to reach final concentrations of 12.5, 25, 37.5, and 50 ppm, each 100 mL per treatment..

2.4. Preservation Treatment

Each treatment was carried out in a closed container containing two milkfish (*Chanos chanos*) weighing approximately 5 g each. The fish samples were immersed in ethanolic extracts of *Rhizophora mucronata* leaves at four concentrations (12.5, 25, 37.5, and 50 ppm), along with a control group without extract. The immersion process was performed at room temperature for 12, 24, 36, and 48 hours, respectively. After the preservation period, the fish samples were drained and analyzed for their protein content. The preservation process of milkfish using ethanolic mangrove leaf extract at different concentrations is shown in Figure 2.

Figure 2. Preservation of milkfish (*Chanos chanos*) using ethanolic extract of *Rhizophora mucronata* leaves at different concentrations (12.5, 25, 37.5, and 50 ppm).

2.5. Protein Determination

The protein content of the fish samples was determined using the Kjeldahl method (AOAC, 2005), which consists of three main stages: digestion, distillation, and titration.

- Digestion: Approximately 0.05 g of fish sample, 1 g of selenium mixture, and 2 mL of concentrated H_2SO_4 were heated until a clear green solution was obtained.
- Distillation: The digested sample was mixed with 80 mL of 50% NaOH, 100 mL of distilled water, and phenolphthalein (PP) indicator, and then distilled until a clear distillate was collected.
- Titration: A 20 mL aliquot of the distillate was collected in an Erlenmeyer flask containing 10 mL of 4% boric acid solution and titrated with 0.02 N HCl until a pale pink endpoint appeared.

The percentage of nitrogen was calculated using the following formula:

$$\%N = \frac{V \times N \times 14.008 \times 100}{sample\ mass \times 1000} \quad ...(1)$$

The percentage of nitrogen obtained was multiplied by a conversion factor of 6.25 to determine the protein content.

$$\text{Protein content (\%)} = \%N \times 6.25 \quad ...(2)$$

2.6. Data Analysis

Protein content data were analyzed using two-way analysis of variance (Two-Way ANOVA) to examine:
(a) the effect of extract concentration (Factor A),
(b) the effect of soaking duration (Factor B), and
(c) the interaction between the two factors (A × B).

The level of significance was set at $\alpha = 0.05$. When significant differences were detected, Tukey's Honest Significant Difference (HSD) post-hoc test was applied. Statistical analyses were performed using SPSS version 26 or R software.

2.7. Ethical and Environmental Considerations

All experimental procedures followed standard laboratory safety protocols and ethical guidelines for the treatment of test animals, ensuring that no suffering was caused. The use of mangrove leaves was conducted sustainably by collecting only fallen leaves or pruned materials from mangrove rehabilitation areas. This approach was intended to establish a quantitative relationship between the concentration of *R. mucronata* extract and soaking duration on the protein content of milkfish, as well as to determine the optimal extract concentration capable of significantly preserving protein compared with the control.

3. RESULTS AND DISCUSSION

Before analyzing the protein content of milkfish, the ethanolic extract of *Rhizophora mucronata* leaves used in this study was first observed for its physical characteristics. The extract appeared as a dark green to

brownish viscous liquid with a distinctive mangrove odor, indicating the presence of phenolic and flavonoid compounds typically found in mangrove species. The appearance of the ethanolic extract is shown in Figure 3.

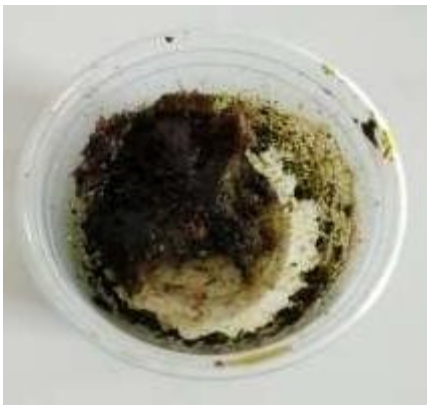

Figure 3. Ethanolic extract of *Rhizophora mucronata* leaves used as treatment in the preservation of milkfish (*Chanos chanos*).

In addition, the ethanolic extract was diluted into several concentrations (12.5, 25, 37.5, and 50 ppm) to be applied to the milkfish samples during preservation. Each concentration showed different color intensities corresponding to the concentration of bioactive compounds. The appearance of the diluted ethanolic extract solutions is presented in Figure 4.

Figure 4. Ethanolic extract solutions of *Rhizophora mucronata* leaves at different concentrations (12.5, 25, 37.5, and 50 ppm).

3.1. Protein Content Test

B Based on the protein content analysis of milkfish conducted using the Kjeldahl method, the data obtained are presented in Table 1 below:

Table 1. Results of Protein Content Test in Milkfish (*Chanos chanos*)

Concentration of Mangrove Extract (ppm)	Preservation Time	Protein Content (%)		Average
		Replication 1	Replication 2	
12.5 ppm	12 h	18.83	17.40	18.115
	24 h	18.15	15.89	17.02
	36 h	15.12	15.57	15.345
	48 h	14.123	12.77	13.4465
25 ppm	12 h	19.07	20.12	19.595
	24 h	18.14	16.96	17.55
	36 h	15.34	15.99	15.665
	48 h	14.33	13.88	14.105
37.5 ppm	12 h	20.07	19.62	19.845
	24 h	19.02	17.52	18.27

	36 h	17.53	17.12	17.325
	48 h	16.18	14.95	15.565
50 ppm	12 h	20.80	20.34	20.57
	24 h	19.87	18.66	19.265
	36 h	18.15	19.62	18.885
	48 h	17.40	17.72	17.56

The data indicate that the higher the concentration applied to the milkfish, the greater the increase in protein content. Furthermore, an analysis of the relationship between soaking time and extract concentration on the protein content of milkfish was conducted. To determine the significance of these effects, an ANOVA test was performed. The results of the ANOVA test are presented in Table 2 below.:

Table 2. Results of ANOVA Test on Protein Content of Milkfish (*Chanos chanos*)

Source	SS	Df	Mean Square	F Value	Sig. (P)
Corrected Model	307.875[a]	19	16.204	12.558	0.000
Intercept	8781.512	1	8781.512	6805.824	0.000
Time	139.608	3	46.536	36.066	0.000
Concentration	159.983	4	39.996	30.997	0.000
Time * Concentration	26.985	12	2.249	1.743	0.148
Error	20.645	16	1.290		
Total	10417.383	36			
Corrected Total	328.519	35			

Based on Table 4.2, the results of the interaction test between concentration and soaking time on protein content showed a *p*-value greater than the significance level (0.148 > 0.05), indicating that there was no significant interaction effect between concentration and time on protein content. However, when analyzed separately, the *p*-value for concentration was smaller than the significance level (0.00 < 0.05), indicating a significant effect of extract concentration on protein content. Similarly, soaking time also had a significant effect on protein content, as the *p*-value was less than α (0.000 < 0.05).

3.2. Protein Content Analysis

The determination of protein content in milkfish preserved with ethanol extracts at various concentrations was carried out using the Kjeldahl method. This method determines the total nitrogen present in amino acids, proteins, and other nitrogen-containing compounds. It is suitable for semi-micro analysis because it requires only a small amount of sample and reagents, as well as a relatively short processing time. The analysis of protein content using the Kjeldahl method generally consists of three main stages:

3.2.1 Digestion

The digestion stage is the decomposition process of nitrogen in the sample using concentrated acid. In this step, approximately 0.05 g of milkfish sample is heated with concentrated sulfuric acid (H_2SO_4) to break down the organic material into its elemental components. The elements carbon (C) and hydrogen (H) are oxidized to form CO, CO_2, and H_2O, while nitrogen (N) is converted into $(NH_4)_2SO_4$. To accelerate the digestion process, a catalyst such as selenium is added because it increases the boiling point of the mixture. The completion of digestion is indicated by the solution turning into a clear green color.

$$R\text{-}C\text{-}COOHNH_3 + H_2SO_4 \rightarrow CO_2 + H_2O + (NH_4)_2SO_4 \quad ...(3)$$

3.2.2 Distillation

At this stage, ammonium sulfate is decomposed into ammonium (NH_3) by adding NaOH until the solution becomes alkaline and is then heated. The released ammonia is subsequently captured by boric acid. To ensure optimal contact between the acid and ammonia, the tip of the distillation tube is immersed as deeply as possible into the boric acid solution. To monitor the condition of the distillate, the indicator Methyl Red (MR) is added. The reaction occurring in this stage is shown in Equation 4 below:

$$(NH_4)_2SO_4+2NaOH\rightarrow 2NH_3+Na_2SO4+2H_2O...(4)$$

3.2.2 Titration

This stage aims to determine the amount of ammonia in the receiving solution. The nitrogen content can be calculated from the amount of ammonium ions present in the solution. In this stage, the distillate is titrated

using hydrochloric acid (HCl). The boric acid reacts with the HCl, and the endpoint of the titration is indicated by a color change from clear yellow to pale pink. The reaction occurring in this stage is shown in Equation (5) below:

$$NH_3 + HCl \rightarrow NH_4Cl \quad ...(5)$$

Based on the quantitative analysis conducted using the Kjeldahl method, the protein content of milkfish samples preserved with ethanol extract of mangrove leaves at concentrations of 12.5 ppm, 25 ppm, 37.5 ppm, and 50 ppm, and soaking durations of 12, 24, 36, and 48 hours, is presented in Figure 5 below.

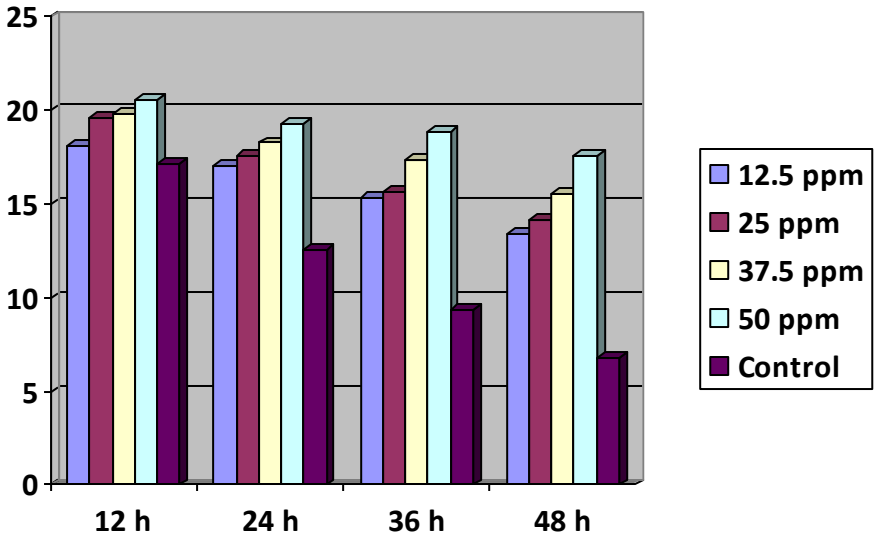

Figure 5. Protein content of milkfish

Figure 5 shows that milkfish preserved with 50 ppm ethanolic extract of *Rhizophora mucronata* leaves for 12 hours exhibited the highest protein content, while the protein levels gradually decreased with longer preservation times across all treatments.

3.3. Analysis of Protein Content Changes in Milkfish After the Addition of Ethanolic Extract of Mangrove Leaves

The results of this study indicate that the increase in the concentration of ethanolic extract of mangrove leaves (*Rhizophora mucronata*) is directly proportional to the increase in protein content of milkfish (*Chanos chanos*) during the preservation process. The treatment with a concentration of 50 ppm produced the highest protein content after 12 hours of preservation compared to other concentrations. This finding suggests that the bioactive compounds in mangrove leaves, particularly flavonoids, phenolics, and tannins, possess the ability to protect protein structures from oxidation and denaturation processes. Basuini et al. [24] reported that the addition of *Avicennia marina* mangrove leaf extract to grey mullet effectively increased total protein levels and slowed protein degradation during storage, supporting the present findings. Similarly, Permatasari et al. [25] found that mangrove leaf extract can function as an effective natural preservative in maintaining the quality of threadfin bream during cold storage.

However, the interaction between extract concentration and preservation time showed no significant effect on protein content ($P = 0.148 > 0.05$). This indicates that extract concentration has a greater influence on protein stability than preservation duration. This result aligns with the findings of Saimima et al. [26] , who reported that *R. mucronata* leaf extract exhibits strong antibacterial activity against *Vibrio harveyi*, mainly due to its phenolic and flavonoid compounds that act as natural antioxidants. Likewise, Linayati et al. [27] demonstrated that mangrove leaf extract effectively inhibits the growth of pathogenic bacteria in the aquaculture system of whiteleg shrimp (*Litopenaeus vannamei*) through strong antibacterial and antioxidant activities. Furthermore, Mulyani et al. [28] reported that *R. mucronata* leaf extract can enhance the immune response of *Cyprinus rubrofuscus* infected with *Aeromonas hydrophila*, reinforcing evidence that the bioactive compounds in mangrove extracts play a role in maintaining protein integrity and the tissue structure of aquatic animals.

In addition to supporting protein stability, several studies have shown that phenolic compounds in mangroves are capable of suppressing lipid oxidation and inhibiting proteolytic activity that can damage fish

tissues during storage [29], [30]. Herefore, the use of ethanolic extract of mangrove leaves has potential as an environmentally friendly natural preservative in the fishery industry, particularly for maintaining the nutritional quality of milkfish, which holds high economic and nutritional value in the Philippines.

This study presents a novel contribution in the utilization of ethanolic extract of *Rhizophora mucronata* leaves as a natural preservative capable of maintaining the protein content of milkfish (*Chanos chanos*) under tropical conditions in the Philippines. Unlike previous studies that focused mainly on the antioxidant and antibacterial activities of mangrove plants, this study provides quantitative evidence that the 50 ppm extract concentration is the most effective in preventing protein degradation during 12 hours of preservation. These findings emphasize the role of phenolic and flavonoid compounds in inhibiting protein oxidation, expanding the practical application of these natural substances in preserving tropical fishery products, and opening opportunities for further research on mangrove-based biomaterials for sustainable seafood industries.

The implications of this study are scientific, economic, and environmental. Scientifically, the findings strengthen the understanding of natural antioxidant mechanisms in preserving fish protein structure and may serve as a reference for developing phytochemical-based preservative formulations. Economically, the application of locally available mangrove leaf extract can reduce production costs and provide an environmentally friendly alternative for fishers and processors in coastal areas of the Philippines. Ecologically, the sustainable use of mangrove resources can promote mangrove ecosystem conservation while enhancing coastal food security by improving the quality and shelf life of milkfish products. However, this study was limited to a single fish species, namely milkfish, and therefore cannot represent all types of fish.

4. CONCLUSION

Based on the findings, it can be concluded that the protein content of milkfish (*Chanos chanos*) increased with higher concentrations of ethanolic extract of mangrove leaves (*Rhizophora mucronata*), indicating the extract's effectiveness in preserving protein during storage. The treatment with a 50 ppm concentration yielded the highest protein level after 12 hours, suggesting that bioactive compounds such as phenolics, flavonoids, and tannins play a key role in protecting protein structures from oxidation and denaturation. However, the interaction between extract concentration and preservation time was not significant ($P = 0.148 > 0.05$), implying that concentration had a stronger effect on protein stability than immersion duration. These findings highlight the potential of *R. mucronata* leaf extract as an eco-friendly natural preservative in maintaining the nutritional quality of milkfish under tropical conditions. Future research should include pre- and post-treatment protein analysis and employ alternative protein determination methods, such as the Lowry or Bradford assay, while also expanding the investigation to other nutritional parameters (lipids, vitamins, and minerals) to gain a more comprehensive understanding of the extract's preservative effects.

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