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FTIR-ATR CHARACTERIZATION AND SCREENING OF ANTIOXIDANT AND ANTI-INFLAMMATORY POTENTIAL OF CHITIN, AND COLLAGEN DERIVED FROM *THUNNUS THYNNUS* AND *SARDINA PILCHARDUS* COPRODUCTS

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Abstract

The problem of fishery coproducts has increased in recent years and has become a global problem influenced by various biological, technical, operational, and socio-economic factors. This work aims to valorize the coproducts of two pelagic species, bluefin tuna (*Thunnus thynnus*) and sardines (*Sardina pilchardus*), by the extraction and characterization of the bioactive substances (Chitin and collagen) by physicochemical and FTIR-ATR analysis, and the in vitro evaluation of their antioxidant and anti-inflammatory potential. From the extraction yield, *T. thynnus* recorded the highest extraction yield for both fractions (chitin and collagen) compared to *S. pilchardus.* The FTIR-ATR spectrum showed bands and peaks corresponding to functional groups of the substances tested. The evaluation of the antioxidant potential showed that sardine and tuna collagen have good antiradical, anti-inflammatory, and anti-hemolytic effects compared to chitin. The results revealed that the bioactive substances (chitin and collagen) extracted from *T. thynnus* and *S. pilchardus* byproducts could have promising applications in the food, pharmaceutical, and nutraceutical sectors.

Keywords: Sardina pilchardus; Thunnus thynnus; Coproducts; Chitin; Collagen.

ХАРАКТЕРИСТИКА МЕТОДОМ FTIR-ATR ТА СКРИНІНГ АНТИОКСИДАНТНОГО ТА ПРОТИЗАПАЛЬНОГО ПОТЕНЦІАЛУ ХІТИНУ ТА КОЛАГЕНУ, ОТРИМАНИХ ІЗ ПОБІЧНИХ ПРОДУКТІВ *THUNNUS THYNNUS* ТА *SARDINA PILCHARDUS*

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Анотація

Проблема побічних продуктів рибальства загострилася в останні роки і стала глобальною проблемою, на яку впливають різні біологічні, технічні, операційні та соціально-економічні фактори. Ця робота має на меті утилізацію побічних продуктів двох пелагічних видів, синього тунця (*Thunnus thynnus*) та сардини (*Sardina pilchardus*), шляхом екстракції та характеристики біоактивних речовин (хітину та колагену) за допомогою фізико-хімічного та FTIR-ATR аналізу, а також in vitro оцінки їх антиоксидантного та протизапального потенціалу. Щодо виходу екстракції, *T. thynnus* показав найвищий вихід для обох фракцій (хітину та колагену) порівняно з *S. pilchardus*. Спектр FTIR-ATR показав смуги та піки, що відповідають функціональним групам досліджуваних речовин. Оцінка антиоксидантного потенціалу показала, що колаген сардини та тунця має хороші антирадикальні, протизапальні та антигемолітичні ефекти порівняно з хітином. Результати показали, що біоактивні речовини (хітин та колаген), екстраговані з побічних продуктів *T. thynnus* та *S. pilchardus*, можуть мати перспективне застосування у харчовій, фармацевтичній та нутрицевтичній галузях.

Ключові слова: *Sardina pilchardus; Thunnus thynnus*; Побічні продукти; Хітин; Колаген.

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Introduction

Currently, annual global fish production is more than 167 million tonnes caught or farmed. These fish can be eaten fresh, dried, frozen, or processed [1]. Many of the world's fish production undergoes processing such as filleting, heading, evisceration, peeling, etc. These processing stages generate a considerable quantity of waste and co-products estimated at 50 % of the weight of fish. These wastes are often directly released into the environment without appropriate treatment, which causes significant environmental problems. The coproducts are mainly composed of heads, viscera, skins, bones, tails, fins, and filleting residues and contain potentially interesting constituents (antioxidants, anti-stress, antihypertensives, collagens, pigments, etc.) [2].

Coproduct recovery routes represent a solution for this waste because, in addition to respecting the environment, they would make it possible to minimize contamination problems and maximize company profits [3]. They consist of transforming them into raw materials or intermediate materials for producing other products. Given the nature of their substances, they can be used for human and animal nutrition in the form of fish meal and oil, nutraceutical, and pharmaceutical products [4].

Technological, cosmetic, and pharmaceutical goods have benefited from the extraction of intriguing chemicals. According to Gaikwad, S., Kim [5], collagen is derived from the skin, bones, cartilage, and scales of fish, whereas omega-3 is collected from the fish's head and viscera. Using a waste management plan that includes turning these byproducts into items that can be sold could be a good way to deal with this situation [6]. Research has demonstrated that regular use of fish-derived supplements can stimulate the production of compounds with a range of health advantages, including immunomodulatory, antihypertensive, and antihyperlipidemic qualities [7]. Global attempts to find new biologically active compounds that create secondary metabolites and macromolecules with antioxidant and anti-inflammatory action are centered on coproducts and their metabolites [8].

This work aims to valorize the byproducts of two pelagic species: bluefin tuna (*Thunnus thynnus*) and sardines (*Sardina pilchardus*). Therefore, the objective is based on the extraction and physicochemical and FTIR-ATR characterization of bioactive substances from sardines (*Sardina pilchardus*) and tuna (*Thunnus thynnus*) coproducts and the *in vitro* evaluation of their antioxidant and anti-inflammatory potential.

Results and their discussion

Extraction yields

The extraction yields and aspects of chitin and collagen of the coproducts of *S. pilchardus* and *T. thynnus* are shown in Table 1. From these results, it was noticed that *T. thynnus* waste recorded the highest extraction yield for both fractions (chitin and collagen) with yields of 14.42 ± 0.26 % and 24.46 ± 1.16 % respectively, compared to the coproducts of *S. pilchardus* (12.31 ± 0.4 % and 19.26 ± 0.38 %). This difference in extraction yield depended on the species, the coproducts used (spines, heads, and viscera), the extraction technique, the diversity of fish species, and the quality of the raw material used [9].

Table 1

Extraction yields and IC_{50} values

	Yields (%)	IC_{50} (mg/m**L**)
Chitin-S	12.31 ± 0.4	0.275
Collagen-S	19.26 ± 0.38	0.26
Chitin-T	14.42 ± 0.26	0.285
Collagen-T	24.46 ± 1.16	0.27
Ascorbic acid	/	0.125

S: Sardines, T: Tuna

Spectroscopic analysis (FTIR-ATR)

The FTIR-ATR spectra of chitin and collagen extracted from *S. pilchardus* and *T. thynnus* are shown in Fig. 1 and 2, respectively. For chitin extracted from sardine and tuna coproducts, the IR-ATR spectrum presents: A broadband appeared at 3272 cm^{-1} and 3270 cm^{-1}, representing the elongation vibration of (O-H) (with H bond). Two absorption peaks at 2916 cm^{-1} and 2847 cm^{-1} indicate the asymmetric stretching vibration of (C-H) of (CH_3). An absorption band at 1624 cm^{-1} and 1625 cm^{-1} represents the stretching vibration of the carbonyl group, C=O of acetamide. Another significant band at 1537 cm^{-1} could also be attributed to the amide functional group's N-H bending and C-N stretching. A peak at 1453 cm^{-1} and 1455 cm^{-1} represents the chitin structure's CH_2 termination and CH_3 deformation. A band was observed at 1040 cm^{-1} and 1029 $cm^{-1,}$ indicating the asymmetric stretching of C-O in the phase ring (saccharide ring). The alpha and beta forms of the extracted chitin can be distinguished by IR analysis. In β-chitin, a single band can be observed at 1620–1650 cm^{-1} [10]. Thus, the acetylation degree parameter (DA%) characterizes ß-chitin with a value exceeding 80 % [11]. The degree of

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acetylation (DA%) of chitin was an important parameter because it influenced all of the physicochemical properties of the polymer. Theoretically, the DA value of pure chitin was 100 % [12]. In this study, the DA value of chitin obtained from the coproducts of *S. pilchardus* and *T. thynnus* was 81.19 % and 88.20 %, respectively. This shows that the FTIR spectrum of the product obtained after deproteinization and demineralization of sardine and tuna coproducts was successfully transformed into chitin. Referring to the FTIR-ATR spectrum and the degree of acetylation of chitin extracted from sardine and tuna coproducts, it was proven that the chitin obtained was in the β form. Among the advantages of ß-chitin was its high reactivity and affinity with solvents [13; 14].

For collagen extracted from sardine and tuna coproducts, the FTIR-ATR spectrum presents: A mean band appeared at 3304 and 3268 cm^{-1} for the intermolecular O-H bond with hydrogen bridge, and can represent the NH_2 group (peak ≈3230 cm^{-1}). A medium band appeared at 2924 and 2923 cm^{-1} for the C-H bond in the asymmetric elongation of CH_2. A middle band appeared at 2309 and 2317 cm^{-1}, representing the carboxyl group COOH. A strong peak at 1645 and 1632 cm^{-1}, which indicates the C=O group. It was related to the secondary structure of the protein. A strong peak at 1518 and 1537 cm^{-1} indicated the N-H group by in-plane deformation. It represented the amide II group resulting from an N-H bending combined with a C-N stretching vibration [15]. Peaks between 1224 and 1454 cm^{-1} allow the creation of a helical morphology [16]. Amide III identified the absorption resulting from the agitated vibrations of the CH2 groups of the proline side chains and glycine support, as well as the gathering peaks of C–N stretching, N–H vibrations, and distortion emanating from the amide connection [17].

Aboudamia et al. [18] reported a strong FTIR peak at 1454 cm^{-1} for the carbonate group (CO_3^{2-}) in sardine coproducts, indicating asymmetric stretching, with medium C-O elongation bands at 1079 and 1081 cm^{-1}. Ampitiya et al. [19] identified tuna collagen FTIR peaks for amide A (3298.81 cm^{-1}), amide B (2921.57 cm^{-1}, linked to CH_2 asymmetric stretching, per Verdolino et al. [20], and amides I, II, and III, characteristic of collagen type I [21]. Jayasundara et al. [22] confirmed these amide bands in Thunnus collagen extracts as distinctive FTIR spectra.

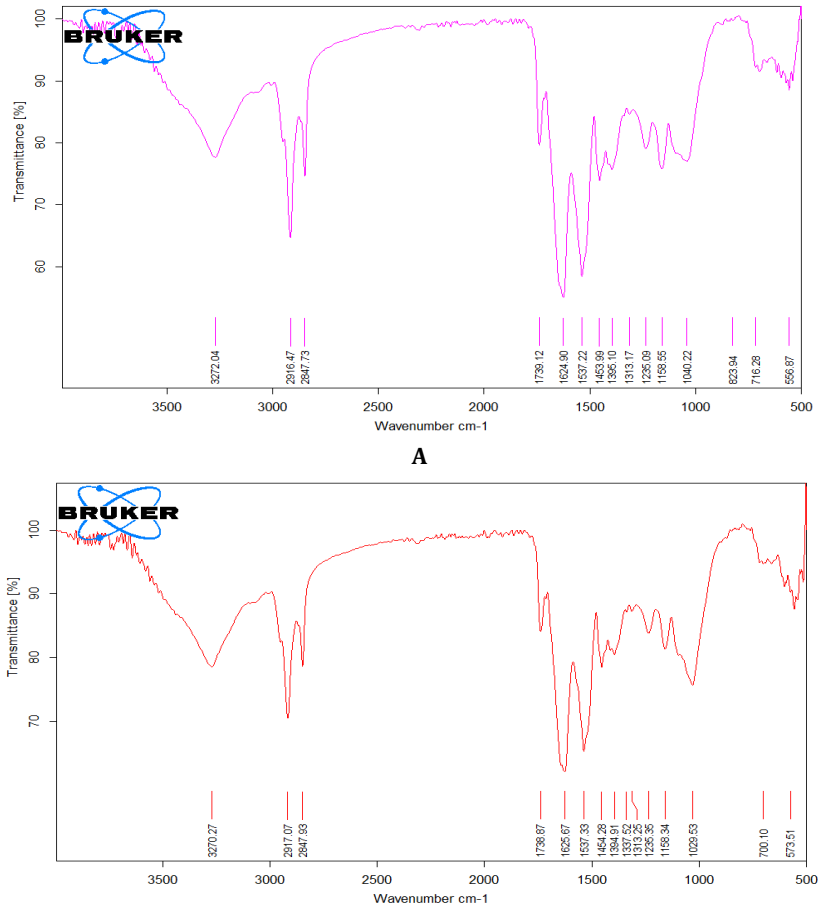

Fig. 1. FTIR spectra of chitin extracted from coproducts of *S. pilchardus* (A) and *T. thynnus* (B)

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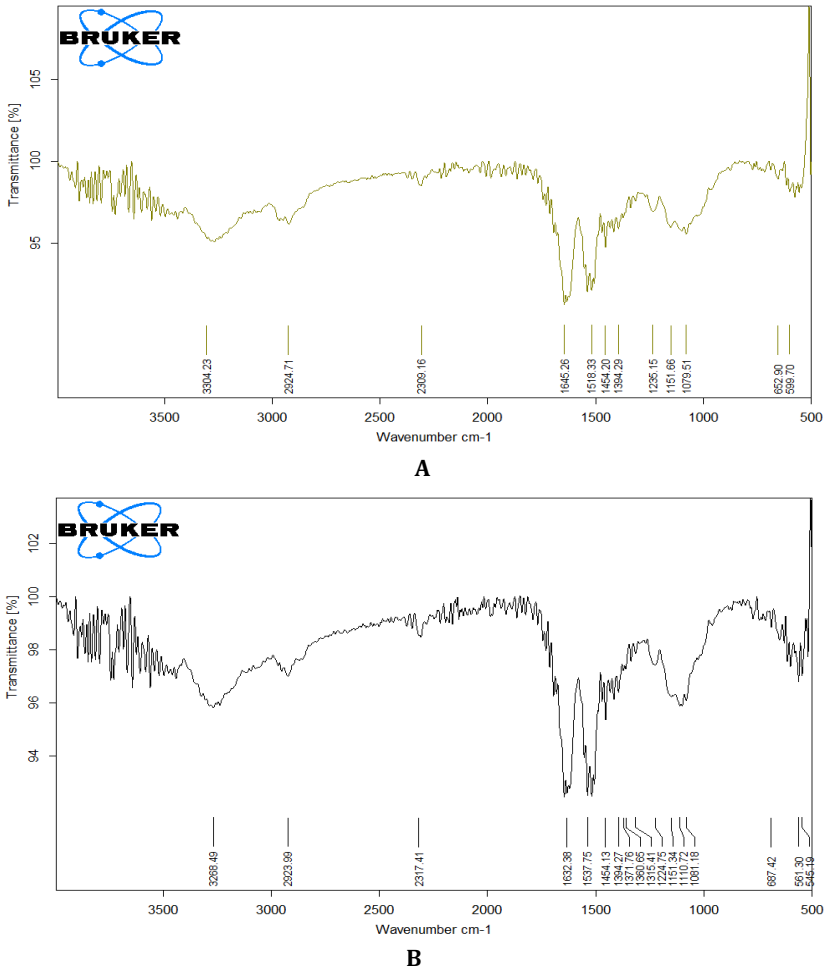

Fig. 2. FTIR spectra of collagen extracted from coproducts of *S. pilchardus* (A) and *T. thynnus* (B). DPPH free radical scavenging

The antioxidant activity profile showed that the three fractions tested have a dose-dependent antiradical activity; that is to say, the increase in the concentration increased the percentage of inhibition (Table 2). The antiradical capacity was determined by comparison with the activity of a reference antioxidant compound (ascorbic acid). From the results obtained, it was noticed that for the concentration of 1000 µg/ml, the collagen of *S. pilchardus* coproducts showed the highest percentage reduction (84.39 ± 0.72 %) compared to other fractions. It should be mentioned that ascorbic acid remains more active than the fractions tested, with an inhibition percentage of 96.63 ± 0.98 %. The concentration necessary to inhibit 50 % of the radical DPPH was calculated by linear regression of the inhibition percentages calculated as a function of different concentrations of the extracts. The IC_{50} values of chitin and collagen of the coproducts of *S. pilchardus* and *T. thynnus* are shown in Table 2.

Table 2

Antiradical activity (%) of chitin and collagen

		125	250	500	1000	IC_{50} (mg/ml)
Sardine	Chitin	17.29 ± 1.03^{d}	40.40 ± 0.95^{c}	54.12 ± 1.71^{b}	66.02 ± 0.43^{ab}	0.275
	Collagen	24.35 ± 0.97^{ab}	51.25 ± 2.30^{ab}	63.41 ± 0.69^{ab}	84.39 ± 0.72^{ab}	0.26
Tuna	Chitin	25.30 ± 0.4^{b}	37.31 ± 1.09^{b}	49.42 ± 0.43^{b}	68.34 ± 0.39^{b}	0.285
	Collagen	29.56 ± 0.44^{ab}	46.56 ± 0.58^{ab}	65.53 ± 0.58^{ab}	80.57 ± 0.32^{ab}	0.27
Ascorbic acid		46.11 ± 0.39^{d}	69.90 ± 1.98^{c}	83.21 ± 2.35^{b}	96.63 ± 0.98^{a}	0.125

Values were presented as mean ± SD of three measurements. Statistical significance was assessed by one-way analysis of variance ANOVA coupled with Tukey's post hoc test. According to Tukey's post hoc test, different letters (a, b, c, and d) in a column indicate a significant difference ($p < 0.05$) between groups.

The collagen of *S. pilchardus* and *T. thynnus* presented the highest antioxidant activity compared to the other fractions, with an IC_{50} value equal to 0.26 mg/ml and 0.27 mg/ml, respectively. However, ascorbic acid showed the lowest IC_{50} value (0.125 mg/ml). Guedes et al. [23] confirmed this by using *Sardina pilchardus* co-products as a source to produce antioxidants. Radical scavenging assays demonstrated that *S. pilchardus* coproducts effectively neutralize peroxide, hydroxyl, and nitric oxide radicals. According to Borges et al. [24], concentrations of 0.5 and 4 mg/ml were tested to evaluate the protective role of sardine coproducts in situations of induced oxidative stress. Yates et al. [25] showed that *Sardina pilchardus* coproducts reduced oxidative stress and neuroinflammation in several ways.

According to the results of Cardeira et al. [26], the coproducts of *S. pilchardus* exhibited the highest ORAC value (1.94 ± 0.08 μmol TEAC/mg) in the radical absorption capacity test of oxygen. These results were consistent with a previous evaluation of antioxidants by an alternative method (2,2-diphenyl-1-picrylhydrazyl-DPPH), where the coproduct extracts presented the lowest IC_{50} values [27].

However, Manni et al. [28] revealed that the tested coproduct proteins of *S. pilchardus* exhibited remarkable antioxidant activity against DPPH. It displayed the highest DPPH radical scavenging activity with an IC_{50} value of 1.14 ± 0.08 mg/mL. According to Mutamimah et al. [29], the inhibition of tuna coproduct protein against the DPPH radical was 78.44 ± 0.40 % at a 10 mg/mL concentration. They stated that the ability of proteins and their derivatives to inhibit lipid oxidation makes them an essential part of the antioxidant defense of biological tissues.

According to Rachman et al. [30], the evaluation results showed that the antioxidant activity of tuna collagen was higher in the DPPH method. Similar results were also reported by Nurilmala et al. [31] on tuna skin collagen. The DPPH method was suitable for hydrophobic compounds. This finding can be attributed to the raw material properties of tuna skin reported by Suseno et al. [32], which includes 15.50 % hydrophobic amino acids and 14.74 % hydrophilic amino acids of the total amino acids detected.

Tuna skin contains 21.92 %, 9.76 %, and 8.94% of the amino acids glycine, proline, and arginine, respectively, which are also thought to play a role in antioxidant activity. In addition to glycine and proline, which are characteristic of collagen, hydroxyproline is also one of the dominant amino acids in collagen proteins and plays a key role in their structural stability and biological activity [29]. Pratama et al. [33] stated that the increased amount of hydrophobic amino acids (valine, isoleucine, phenylalanine, methionine) in tuna coproducts contributed to increased antioxidant activity. According to Thaha et al. [34], hydrophobic amino acids can prevent the oxidation of macromolecules by donating protons to reactive radicals.

According to Rachman et al. [30], the antioxidant activity of tuna collagen was included in the category of moderate antioxidants (IC50 > 0.20 mg/mL). At the same time, Chi et al. [35] showed that bigeye tuna head proteins have an IC_{50} value of 1.34 mg/mL. Various sources of marine collagen have reported antioxidant activities [36]. Many antioxidant peptides have already been identified, and numerous studies have demonstrated that they positively affect human health and can be applied in the food and cosmetic industries [37].

Peptides made from marine byproducts have been demonstrated in numerous studies to have potent antioxidant qualities. By giving radicals made of unsaturated lipids an electron or a hydrogen atom, the antioxidant can stop oxidation. By eliminating radical initiators or intermediates from the medium and inactivating metal catalysts, it can prevent these radical chain reactions [38].

Inhibition of protein denaturation

According to the results (Table 3), there was a proportional relationship between the concentration of the extracts tested and the percentage of inhibition of protein denaturation. In addition, it was shown that *S. pilchardus* collagen presented a percentage of inhibition of BSA denaturation ranging from

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29.81 ± 0.61 to 86.03 ± 0.19 % for concentrations varying from 125 to 1000 µg/ml. It was followed by *T. thynnus* collagen with a value of 80.73 ± 0.65 % for the concentration 1000 µg/ml. The comparison with Ibuprofen showed that the latter presented a higher percentage of inhibition (91.10 ± 2.1 %). For the coproducts of the two species studied, chitin presented an average inhibition rate of 60.96 ± 0.47 and 62.79 ± 2.05 % for *S. pilchardus* and *T. thynnus*, respectively, compared to other fractions.

Table 3

The inhibition percentage (%) of BSA by chitin and collagen.

		125	250	500	1000
Sardine	Chitin	21.25 ± 0.63^{bc}	38.57 ± 0.53^{b}	53.69 ± 0.85^{ab}	60.96 ± 0.47^{ab}
	Collagen	29.81 ± 0.61^{c}	55 ± 0.72^{b}	76.53 ± 0.43^{ab}	86.03 ± 0.19^{ab}
Tuna	Chitin	22.44 ± 1.23^{bc}	30.18 ± 0.89^{b}	48.93 ± 0.43^{ab}	62.79 ± 2.05^{ab}
	Collagen	31.20 ± 0.61^{c}	58.55 ± 1.32^{b}	73.37 ± 0.35^{ab}	80.73 ± 0.65^{ab}
Ibuprofen		36.78 ± 0.38^{a}	60.98 ± 0.3^{a}	78.02 ± 1.11^{b}	91.10 ± 2.1^{c}

Values were presented as mean ± SD of three measurements. Statistical significance was assessed by one-way analysis of variance ANOVA coupled with Tukey's post hoc test. According to Tukey's post hoc test, different letters (a, b, c, and d) in a column indicate a significant difference ($p < 0.05$) between groups.

The study by Yates et al. [25] showed that the anti-inflammatory effect of *S. pilchardus* coproducts was more marked after 12 hours of incubation. The ability to help reduce inflammation was comparable to that of piroxicam (an anti-inflammatory drug), which was even more effective than the drug after 12 and 24 hours. According to the results presented by Zajdel et al. [39], there was a reduction in the TNF-α level in cells exposed to *S. pilchardus* coproducts. These inhibitory effects were associated with a decrease in the gene expression of these cytokines. According to Guedes et al. [23], extracts derived from *S. pilchardus* co-products significantly reduced the levels of all pro-inflammatory mediators studied. According to the study by Sharma et al. [40], Thunnus coproduct extract showed significant anti-inflammatory activity, which may be due to the inhibition of inflammatory mediators by the action of steroids. Synergistic effects between hydrophobic and hydrophilic chemicals from different fish sources and anti-inflammatory qualities have been observed by other authors.

On the other hand, the study by Bae et al. [41] added that the pharmacological activities of chitin have shown its anticancer and anti-inflammatory potential to accelerate wound healing. Fernandes et al. [42] demonstrated that the anti-inflammatory activity of chitin in the carrageenan-induced paw edema method was not only dose-dependent but also molecular weight-dependent. In addition, oral chitin administration was effective against intestinal inflammation and mortality in the acute colitis model. Furthermore, several studies suggested anti-inflammatory, antioxidant, neuroprotective, and cardioprotective activities of chitin and its derivatives [43].

Anti-hemolytic activity

For the in vitro assessment of the anti-hemolytic characteristics, the hypotonic hemolysis method was used since the erythrocyte membrane is similar to the lysosomal membrane, and its stabilization suggests that the studied extract can similarly stabilize lysosomal membranes. Limiting the inflammatory response requires stabilizing the lysosomal membrane because it stops the release of lysosomal components from active neutrophils, such as proteases and bactericidal enzymes, which, when released extracellularly, induce more inflammation and tissue damage [40]. Table 4 shows the percentage of protection against hypotonic hemolysis.

From the values illustrated in Table 4, it can be noted that the percentage of protection against hemolysis presented a proportional relationship with the concentration of chitin and collagen tested. It was pointed out that for the concentration of 1000 µg/ml, *T. thynnus* collagen recorded a protection value equal to 77.39 ± 0.71 %, followed by *S. pilchardus* collagen (58.08 ± 0.65 %). On the other hand, it was mentioned that

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aspirin recorded the highest percentage of protection (88.18 ± 1.56 %) for a concentration of 1000 µg/ml. For chitin, it presented a low protection rate with a value of 60.15 ± 0.42 and 61.70 ± 0.63 % for *S. pilchardus* and *T. thynnus*, respectively.

Table 4

Protection percentage (%) of red blood cells by chitin and collagen

		125	250	500	1000
Sardine	Chitin	12.96 ± 0.28^{d}	22.87 ± 0.035^{c}	40.04 ± 0.86^{cd}	60.15 ± 0.42^{cd}
	Collagen	12.34 ± 1.47^{d}	20.09 ± 0.32^{a}	33.89 ± 0.94^{bc}	58.08 ± 0.65^{bc}
Tuna	Chitin	14.86 ± 0.26^{d}	24.72 ± 0.03^{c}	41.90 ± 0.57^{cd}	61.70 ± 0.63^{cd}
	Collagen	18.3 ± 1.27^{d}	36.58 ± 0.35^{a}	53.06 ± 0.24^{bc}	77.39 ± 0.71^{bc}
Aspirin		38.1 ± 0.92^{d}	57.88 ± 0.73^{c}	74.35 ± 0.25^{b}	88.18 ± 1.56^{a}

Values were presented as mean ± SD of three measurements. Statistical significance was assessed by one-way analysis of variance ANOVA coupled with Tukey's post hoc test. According to Tukey's post hoc test, different letters (a, b, c, and d) in a column indicate a significant difference ($p < 0.05$) between groups.

These results were confirmed by the study of Azeem et al. [40], where they found that the protection of red blood cells by Thunnus extracts was concentration dependent. They demonstrated that Thunnus extracts at concentrations of 100, 200, 300, and 400 mg/ml exhibited stabilization of the membranes of the red blood cells tested. *S. pilchardus* coproducts concentrations of 0.5 and 4 mg/ml were able to shield cells from harm, with fibroblasts showing a greater protective effect, according to Borges et al. (2014). Nuclear factor-erythroid-2-related factor 2 (Nrf2), a transcription factor that was also activated by incubation, was essential for the coordinated activation of several genes encoding cytoprotective and stress-responsive enzymes and associated proteins.

Experimental part

Samples

The experimental study was carried out on coproducts from two species of pelagic fish: sardines (*Sardina pilchardus*) and tuna (*Thunnus thynnus*). Sampling was carried out during February 2024 in the region of Salamandre (Mostaganem, Algeria) (35° 56′ 00″ north, 0° 05′ 00″ east) for the sardine species and in the region of Beni Saf (Ain Temouchent, Algeria) (35° 18′ 8″ North, 1° 23′ 1″ West) for the tuna species.

Chitin extraction

The chitin extraction method was based on the protocol of Boarin Alcalde and Graciano Fonseca [44]. Fish waste was dissolved in HCl 0.5 M at a 1:20 (w/v) ratio for 60 min with constant stirring at room temperature. Then, rinse until neutral with distilled water to remove excess HCl in the treated product, and finally dry at 30°C for 12 h. The demineralized waste was cleaned of remaining proteins with 100 mL of NaOH 1 % solution at a 1 : 10 (w/v) ratio. The extraction time was approximately 180 minutes at 50 °C with constant stirring. The residue was washed with distilled water to reach neutrality and then dried in the oven at 30 °C for 12 h. The product obtained was chitin.

Collagen extraction

The acid-soluble collagen extraction process was carried out according to the method described by Liu et al. [45]. Fish waste was treated with NaOH 0.1 N with a ratio of 1 : 10 (w/v). The mixture was stirred for 6 h, and the solution was changed every 2 h. The residue was washed with water until the pH was neutral. Fats were extracted from deproteinized waste with 10% butanol at 1:10 (w/v). The mixture was stirred for 18 h, and the solution was changed every 6 h. The residue was washed to remove traces of the solvent. Fish waste was treated with 0.5 M acetic acid at a ratio of 1:30 (w/v) and stirred for 24 h at a temperature of 4 °C. The solution was changed every 2 hours. The mixture was filtered, and the filtrate was precipitated with NaCl. The precipitate was collected and centrifuged at 10,000 rpm/2 h. The pellet was lyophilized, and the collagen extract was stored at 4°C.

Spectroscopic analysis (FTIR-ATR)

The analysis was carried out on a BRUKER ALPHA spectrometer equipped with an ATR stage with a diamond crystal triglycine sulfate detector and controlled by the Opus v 7.0 122 software. 1 g of the chitin or collagen sample was directly deposited on the reflection crystal of the Platinum-ATR accessory. The acquisitions were performed by performing 200 scans between 4.000 and 500 cm^{-1} with a resolution of 1 cm^{-1}. For the chitin sample, the degree of acetylation (DA% %) was calculated according to the formula described by Czechowska-Biskup et al. [46]:

$$DA\ (\%) = [A1655/A3450] \times 115$$

Where A1655 was the absorbance of chitin at the wavelength of 1655 cm^{-1}, and A3450 was the absorbance of chitin at 3450 cm^{-1}.

DPPH free radical scavenging

The DPPH scavenging properties of the products were evaluated using the method of Wei et al. [47]. Samples (chitin, collagen) at 125, 250, 500, and 1000 µg/ml were combined with 2 mL of methanolic DPPH solution (180 µmol/L) and incubated for 30 minutes at room temperature. The residual DPPH radical's absorbance was then measured at 517 nm in relation to a blank. Each sample was evaluated with three duplicates, and the antiradical effect was determined using the following equation:

$$\text{Percentage inhibition (\%)} = (\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}) / \text{Abs}_{\text{Control}} \times 100$$

Inhibition of protein denaturation

The inhibitory activity of bovine serum albumin denaturation was exploited in the study by Hu et al. [48]. 50 µL of an extract (chitin, collagen) solution (125, 250, 500, and 1000 µg/ml) and 950 µL of a bovine serum albumin solution (dissolved in 0.01 M PBS, pH 7.4, 1 %, w/v) were used to create the reaction solution. In order to induce protein denaturation, all reaction solutions were stabilized at 37 °C for 15 minutes and then heated to 90 °C for 20 minutes. At room temperature (25 °C), 660 nm was used to measure turbidity. As a control, distilled water was utilized rather than the sample. The rate of denaturation inhibition was determined using the following formula:

$$\text{Percentage inhibition (\%)} = (\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}) / \text{Abs}_{\text{Control}} \times 100$$

Anti-hemolytic activity

To evaluate the protective effect of chitin and collagen extracts against hypotonic lysis-induced damage in human erythrocytes, an in vitro hemolysis assay was conducted following Girish et al. [49]. Blood from a healthy volunteer was collected in heparinized tubes, centrifuged at 1500 rpm for 10 min to remove plasma and buffy coat, and erythrocytes were washed thrice with 10 volumes of PBS (10 mM, 150 mM NaCl, 1.9 mM NaH2PO4, 8.1 mM Na2HPO4, pH 7.4) at 1500 rpm for 5 min. A 10 % v/v erythrocyte suspension was prepared in PBS, stored at 4 °C, and used within 6 h. Aliquots of 0.5 mL extract (125, 250, 500, 1000 µg/mL in 0.2 M phosphate buffer) were mixed with 0.5 mL erythrocyte suspension and 0.5 mL hyposaline solution, incubated at 37 °C for 30 min, and centrifuged at 9000 rpm for 20 min. Supernatant hemoglobin was measured at 560 nm using a UV-Vis spectrophotometer. Aspirin served as the standard, and distilled water replaced the extract for 100 % hemolysis control. Protection percentage was calculated using the provided formula:

$$\text{Percentage of protection} = (\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}) / \text{Abs}_{\text{Control}} \times 100$$

Statistical analysis

Values were reported as mean ± SD (Standard Deviation). The data were investigated with ANOVA analysis. The Differences were recognized as significant at $P < 0.05$. The application of ANOVA was performed using the ANOVA function in R (R Core Team, 2024). The HSD.test function, under the Agricolae package [50], was used to apply the Tukey test.

Conclusions

All of these results showed that the bioactive substances extracted from the coproducts of bluefin tuna (*Thunnus thynnus*) and sardine (*Sardina pilchardus*) have an interesting antiradical and anti-hemolytic activity and, therefore, present considerable therapeutic interest as an alternative compound or therapeutic support for the prevention of inflammation, oxidative stress, and cardiovascular pathologies.

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