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Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. *Mol Syst Biol* 4: 187. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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Pharmacognostic characterization and biological activities of *Aponogeton crispus* and *A. rigidifolius*

RANUJA SARVESWARAN¹, W. J. A. BANUKIE JAYASURIYA^{1,*}, BHAVANTHA DIAS¹, ISURU
KARIYAWASAM², PRIYANI HETTIARACHCHI³, SUGANDHIKA SURESH⁴

¹Department of Pharmacy and Pharmaceutical Sciences, Faculty of Allied Health Sciences, University of Sri Jayewardenepura.
Nugegoda, Sri Lanka. Tel.: +94-11-2758000, *email: banukie@sjp.ac.lk

²Department of Botany, Faculty of Applied Sciences, University of Sri Jayewardenepura. Nugegoda, Sri Lanka

³Department of Biological Sciences, Faculty of Applied Sciences, Rajarata University of Sri Lanka. Mihintale 50300, Sri Lanka

⁴Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura. Nugegoda, Sri Lanka

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Abstract. Sarveswaran R, Jayasuriya WJAB, Dias B, Kariyawasam I, Hettiarachchi P, Suresh S. 2025. Pharmacognostic characterization and biological activities of *Aponogeton crispus* and *A. rigidifolius*. *Asian J Nat Prod Biochem* 23: 1-10. The potential anti-inflammatory effects of the plant family Aponogetonaceae have yet to be widely studied despite the existing ethnomedicinal evidence. This study aimed to investigate the pharmacognostic characteristics and pharmacological activities of *Aponogeton crispus* Thunb. and *Aponogeton rigidifolius* H. Bruggen plants. Anti-inflammatory and anti-hyperglycemia-related bioactivities were evaluated using well-established in vivo and in vitro methods. The safety profiles of the aqueous plant extracts were investigated in acute toxicity and chronic toxicity studies. Significance among different test groups in each assay was analyzed statistically. Reducing sugars, amino acids, alkaloids, flavonoids, and polyphenols were found in the preliminary phytochemical screening. In pharmacological assessments, aqueous extracts of *A. crispus* at 120 mg/kg showed the highest inhibition of carrageenan-induced paw edema, indicating its significant therapeutic potential. The percentage reduction in acetic acid-induced writhes was $46.48 \pm 3.46\%$ for *A. crispus* and $54.22 \pm 2.10\%$ for *A. rigidifolius*. Both extracts significantly reduced the rat body temperature at the third and fourth hour in brewer's yeast-induced pyrexia. Moreover, both plants showed appreciable safety profiles in 2,000 mg/kg dose. The study concludes that both *A. crispus* and *A. rigidifolius* plants possess explicit pharmacognostic characteristics, show significant therapeutic effects against the cardinal signs of inflammation and hyperglycemia, also exhibit no in vivo toxicities.

Keywords: Anti-hyperglycemic, anti-inflammatory, aquatic plants, in vivo, pharmacognosy

INTRODUCTION

The plant family Aponogetonaceae comprises only a single genus, *Aponogeton*, and consists of approximately sixty freshwater monocotyledons, including *Aponogeton natans* Engl. & K. Krause, *Aponogeton crispus* Thunb., *Aponogeton rigidifolius* H. Bruggen, *Aponogeton madagascariensis* (Mirb.) H. Bruggen, *Aponogeton jacobsonii*, *Aponogeton appendiculatus*, and *Aponogeton undulatus* Roxb. which are distributed mainly in the tropical or subtropical regions of the world (Manawaduge and Yakandawala 2018). *Aponogeton crispus* and *A. rigidifolius* are two interesting species found in Sri Lanka with the latter being native. *Aponogeton crispus* normally grows in freshwater tanks, lakes, and ponds, whereas *A. rigidifolius* habitats in slow or fast-running rivers, mostly in wet forests (Wijesundara and Shantha Siri 2004). The genus *Aponogeton* was found to be used in folkloric medicine to treat wounds, snake bites, diarrhea, acne, and jaundice (Chowdhury et al. 2011). Flowering spikes and young shoots of the plants from *Aponogeton* species are consumed as a popular green leafy vegetable (Misra et al. 2012). To alleviate itchiness caused by food allergies, *A. crispus* rhizome is ground with ghee, and the paste is applied over the skin. Flowers of *A. crispus* are specially

used to treat *mhadhumehaya* (diabetes) in Ayurveda (Samarasekara 2002). It was found that the plant *A. rigidifolius* also has medicinal and nutritional benefits that are closely related to *A. crispus* (Weragoda 1994). In addition to the two species under the study, other *Aponogeton* species (e.g., *A. monostachyon*) are used in Siddha medical system to prepare Choorna medicines for skin diseases (Priyadharshini et al. 2019). Some related species, such as *A. appendiculatus*, *A. natans*, and *A. undulatus*, have demonstrated considerable antioxidant properties in various *in vitro* assays (Chougule et al. 2022). The extracts of *A. undulatus* and *A. madagascariensis* have further shown antitumor potential against various cancer cell lines (Islam et al. 2015; Gunawardena et al. 2021). The anti-diabetic properties of *A. natans* leaf extract were evaluated by Dash and co-workers using alloxan-induced diabetic rats; significant reduction in blood glucose levels have been recorded (Dash et al. 2014). *Aponogeton madagascariensis* has been extensively studied in botanical investigations of cell culture and genetic techniques due to its interesting anatomical and physiological characteristics (Rowarth et al. 2021).

Although the phytochemical composition of *A. natans* and *A. appendiculatus* has been extensively studied, the information on the phytoconstituents of *A. crispus* and *A.*

rigidifolius is inadequate in the literature (Chougule et al. 2022). The physicochemical and other pharmacognostic data on these plants were also described insufficiently despite the fact that these plant materials can be adulterated easily by adding either other plants or any foreign material to increase the mass or strength (Seethapathy et al. 2014). Inflammatory conditions involve the production of signaling biomolecules that will encourage the inflamed body area to receive more blood flow and immune cell activation. These inflammatory mediators, however, make pain receptors more sensitive and cause pain sensations. They will disrupt the physiological activity of insulin receptors in muscle and liver tissues and reduce cellular glucose uptake, which ultimately leads to hyperglycemia. Furthermore, body temperature and thrombolytic mechanisms may also be modulated through the inflammatory mediators (Soares et al. 2023). Acute and chronic assessment of toxicity is significant for the safety evaluation of herbal medicines as they have become major aspects in regulatory affairs of ethnomedical systems currently (Mukherjee et al. 2021). Therefore, elaborated investigations on the pharmacognostic characterization and pharmacology of these plant species are needed to promote their use as primary medicines as well as functional food. Considering this background, the present study focused on comparatively evaluating the pharmacognosy, biological effects, and safety profiles of *A. crispus* and *A. rigidifolius* to serve as potential medicinal agents, especially over cardinal signs of pathophysiological inflammation, using various in vivo and in vitro methods (Ribaldone et al. 2019). Additionally, an attempt has been made to scientifically validate the ethnomedicinal use of *A. crispus* as a remedy for diabetes.

MATERIALS AND METHODS

Ethical approval

Ethical clearance was obtained from the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka, under approval certificate no. 43/17 and 46/18 for animal studies and human-involved investigations, respectively. All the information sheets and consent forms were reviewed and approved.

Plant materials

Aponogeton crispus and *A. rigidifolius* plants were collected from the wet zone (6.9061N, 79.9630E and 6.3731N, 80.3575E, respectively) of Sri Lanka and were prepared into herbarium specimens. Then these were authenticated by National Herbarium, Peradeniya, Sri Lanka under the voucher numbers 6/01/H/03 and NH/BOT/4/2019-59, respectively.

Pharmacognostic analysis

The analytical methods described in the literature were used in the pharmacognostic standardization (Kumar et al. 2018). In the macroscopic study, morphological descriptions of different plant parts of *A. crispus* and *A.*

rigidifolius were studied through direct visual observation or with the assistance of a magnifying lens (5x). Freehand cross sections of plant tissues (leaf and petiole) of both plants were stained with safranin. Detailed anatomy was observed under the compound microscope (Olympus, Japan). Structures and features in the cut sections of both plants were recorded using photomicrographs. The petiole parts of both of the plants were powdered separately and then treated with chloral hydrate solution for the microscopic powder analysis performed under the compound microscope. Structures and features in the powders of both plants were recorded using photomicrographs.

Physicochemical analysis

Standard analytical methods were used in the physicochemical standardization (moisture content, total ash, water-soluble ash content, acid-soluble ash content, extractable matter content) of fresh parts of *A. crispus* and *A. rigidifolius* (Sebastian and Nirmal 2020). Where necessary, the hot air oven (Memmert, Germany), muffle furnace (Hobersal, Spain), and analytical balance (Precista, Switzerland) were used.

Phytochemical screening

Qualitative phytochemical screening

A 20.0 g aliquot of air-dried, coarsely powdered flower and stalk samples of both *A. crispus* and *A. rigidifolius* were separately refluxed with dichloromethane for 1.5 hours. The contents were filtered, and the remaining solid residue was refluxed again with ethanol and distilled water successively for the same duration of time. Qualitative phytochemical screening was then carried out on the syrupy material left after evaporating each extract.

In brief, Benedict's test for reducing sugars (1 mL of the sample + 3 drops of Benedict's reagent and heated for 3 minutes), ninhydrin test for amino acids (1 mL of the sample + 3 drops of 0.25% ninhydrin solution and heated for 3 minutes), biuret test for proteins (1 mL of the sample + 3 drops biuret solution and heated for 3 minutes), alcoholic ferric test for tannins (1 mL of the sample + 10% alcoholic ferric chloride), Keller-Kiliani test for cardiac glycosides (2 mL of the sample + ferric chloride in glacial acetic acid + concentrated sulphuric acid), frothing test for saponins (2 mL of the sample was vigorously shaken with 2 mL of distilled water for 60 seconds), Mayer's and Wagner's tests for alkaloids (2 mL of each sample + 3 drops of Mayer's and Wagner's reagents separately), cyanidin test for flavonoids (2 mL of the sample + hydrochloric acid and magnesium), Liberman-Burchard test and Salkowski test for steroids and triterpenoids, sulphuric acid test for quinones (1 mL of the sample + 1 mL of sulphuric acid), hydroxide test for coumarins (1 mL of the sample + 2 drops of 1% NaOH and heated for 3 minutes), and hydrochloric acid test for leucoanthocyanins (1 mL of the sample + 0.5 mL of concentrated hydrochloric acid and heated for 3 minutes) were performed as per the established methods (Morsy 2014).

Quantitative determination of total phenolic content

A 20.0 g aliquot of air-dried, coarsely powdered flower and stalk samples of both *A. crispus* and *A. rigidifolius* were separately refluxed with distilled water for 1.5 hours. The contents were filtered, and the remaining solid residue was removed. The total phenolic content of this crude aqueous extract was assessed by the Folin-Ciocalteu method described by Perera and co-workers with some modifications (Perera et al. 2017). In brief, 500.0 μ L of aqueous extract of *A. crispus*, *A. rigidifolius*, and standard gallic acid ($n=4$) at concentrations of 31.25, 62.5, 125, 250, 500 ppm were mixed with 2.5 mL of Folin-Ciocalteu reagent (diluted ten-fold of initial concentration with deionized water) and left to stand for 5 minutes at room temperature after mixing with 2.5 mL of sodium carbonate solution. The final volume was made up to 10.0 mL using deionized water. The absorbance was measured at 765 nm. The phenolic contents of plant materials were expressed as mg of gallic acid equivalent per gram of extract.

Pharmacological studies

Animals

Both male and female adult Wistar rats (age 6-8 weeks) were purchased from the Medical Research Institute (MRI), Sri Lanka. Animals were housed at room temperature ($27\pm 2^\circ\text{C}$) and a 12-hour light/ dark cycle while feeding with food pellets formulated using MRI standards. Clean, fresh water was provided *ad libitum*.

Preparation of extracts for pharmacological studies

The air-dried and powdered flowers and stalks of each plant (*A. crispus* and *A. rigidifolius*) were combined to get two final powder samples separately. Sixty grams of each sample was boiled with 1.9 L of distilled water in separate clay pots, and the final volume was reduced to about 240.0 mL by gentle boiling. Concentrated crude extracts were freeze-dried separately. The required concentrations of the aqueous extracts of *A. crispus* and *A. rigidifolius* were prepared by dissolving freeze-dried extracts in distilled water suitably.

In vivo anti-inflammatory activity in Wistar rats

Healthy, adult male Wistar rats were randomly divided into 7 groups ($n=6$ per group). The animals in group 1 received 1.0 mL of distilled water; this group served as the baseline for comparison. Animals in groups 2 and 3 were orally administered with aqueous extracts of *A. crispus* at doses of 90 mg/kg and 120 mg/kg body weight (b.w.), respectively. Groups 4 and 5 were orally administered with the same doses of *A. rigidifolius*, respectively. Animals in groups 6 and 7 were treated with standard drugs, indomethacin (10 mg/kg) and diclofenac sodium (15 mg/kg), respectively. After 1 hour of administration of the test samples, inflammation underneath the plantar tissue of the rat's left hind paw was induced by subcutaneous injection of 0.1 mL of carrageenan (1%) aqueous suspension under mild anesthesia. The anti-inflammatory activities of test substances were determined by the paw edema method described elsewhere (Jayasuriya et al. 2020).

In vivo antipyretic activity in Wistar rats

Healthy, adult male Wistar rats were randomly divided into 4 groups ($n=6$ per group). The basal rectal temperature of each rat was recorded using a flexible digital thermometer. Pyrexia in rats was induced by injecting a 10.0 mL/kg b.w. dose of brewer's yeast suspension (20% w/v) into the rat dorsum. Eighteen hours after the injection, the rise in rectal temperature was recorded. Since all the subject animals had $\geq 0.7^\circ\text{C}$ (or 1°F) increase in body temperature, all of them were employed in the investigation. The animals in group 1 served as the control group and received 1.0 mL of distilled water. Groups 2 and 3 rats were administered with aqueous extracts of *A. crispus* and *A. rigidifolius* at a dose of 90 mg/kg b.w., respectively. Acetylsalicylic acid (100 mg/kg) was given orally to test animals in group 4. Then, the rectal temperature of the rats was recorded at hourly intervals for 4 hours to determine the antipyretic activity of the test materials (Bhowmick et al. 2014).

In vivo anti-nociceptive activity in Wistar rats

Healthy, adult male Wistar rats were randomly divided into 4 groups ($n=6$ per group). Animals in group 1 served as the control group and received 1.0 mL of distilled water orally. Groups 2 and 3 rats were treated with aqueous extracts of *A. crispus* and *A. rigidifolius* at a dose of 90 mg/kg b.w., respectively. Group 4 was administered with a 100 mg/kg dose of acetylsalicylic acid and considered as the positive control group. After 30 minutes of oral administration of the test substances, writhing was induced by intraperitoneal 0.6% acetic acid injection (10.0 mL/kg). The anti-nociceptive activity was then assessed by cumulatively counting the number of abdominal constrictions produced for 20 minutes after a latency period of 5 minutes from the injection. The percentage reduction of writhes was determined by the method described in the literature. Anti-nociceptive activities of the test materials and controls were thereby reported as the analgesic index calculated by the methods given in the literature (Rahaman et al. 2020).

In vitro thrombolytic activity on human blood

Healthy human volunteers ($n=10$) who had no history of smoking or taking lipid-lowering drugs, oral contraceptives, or anticoagulant medications were selected, and informed consent was obtained for the study. Then, 6.0 mL of venous blood was withdrawn from each of them, and 2.5 mL of blood volume was transferred to pre-weighed sterile microcentrifuge tubes. Tubes were incubated at 37°C for 45 minutes, and the serum was completely removed from the tube after that. Then, 100.0 μ L quantities of aqueous extracts of *A. crispus* and *A. rigidifolius* in different concentrations (45 mg/mL, 90 mg/mL, 135 mg/mL, and 180 mg/mL) were added to each of the tubes ($n=4$). As the positive control, 100.0 μ L of streptokinase (30,000 IU) was used, and the same amount of sterile distilled water was added to the control. After incubating all tubes at 37°C for another 90 minutes, the supernatant liquid was removed without disturbing the clot. These tubes were weighed again to determine the clot

weight. Results were produced as a percentage of clot lysis (Bhowmick et al. 2014).

In vivo hypoglycemic activity in healthy Wistar rats

The hypoglycemic activity was studied using only the different concentrations of the aqueous extract of *A. crispus*. Healthy, adult male Wistar rats were fasted overnight and randomly divided into 6 groups (n=6 per group). The animals that received 1.0 mL of distilled water were included in group 1, the control group. Animals in groups 2-5 were orally administered with an aqueous extract of *A. crispus* at respective doses of 22.5, 45, 90, and 180 mg/kg b.w. The animals in group 6 were treated with the standard drug metformin (15 µg/kg). After 30 minutes of administration of the test substance, serum glucose levels were raised by loading glucose orally at a dose of 3 g/kg b.w. in all groups. After 90 minutes, blood was collected from the lateral tail vein, and the serum glucose levels were determined using the glucose reagents kits (Biolabo reagents, France). The optimum time of the hypoglycemic activity of the aqueous extract of *A. crispus* (90 mg/kg b.w.) was determined by evaluating serum glucose levels of a separate group of rats at 30-minute intervals from the glucose loading (Jayasuriya et al. 2012).

Evaluation of safety profile

The safety profiles of *A. crispus* and *A. rigidifolius* were assessed according to the guidelines of the Organization for Economic Co-operation and Development (OECD 2002, 2018).

Acute toxicity

Fifteen male and fifteen female Wistar rats were randomly divided into three groups (n=10 per group). Group 1 animals were served as the controlled group and received distilled water. Groups 2 and 3 were treated with 2,000 mg/kg b.w. single doses of aqueous extracts of *A. crispus* and *A. rigidifolius*, respectively. At the end of 14 days, biochemical parameters of the animal, i.e., serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total protein, and creatinine were assessed. All the animals were then sacrificed to determine the relative organ weight percentages.

A histopathological study was conducted to determine whether there were alterations in tissue samples of the heart, kidney, and liver. In brief, rat tissues were cleaned with normal saline and fixed in a 10% formalin solution. Then, the tissues were cleaned thorough with distilled water and processed (Microm STP, United Kingdom) over 24 hours. Tissues were sliced into 4-5 µm thick sections using a microtome, dewaxed with xylene and hydrated through a descending series of ethanol to water. After staining with hematoxylin and eosin, the animal tissues were cover slipped using dibutylphthalate polystyrene xylene (DPX) as the mountant. Finally, slides were microscopically examined for histopathological changes.

Sub-chronic toxicity

Wistar male and female rats were randomly divided into three groups (n=10 per group). The first group was the control and received distilled water. In comparison, the other two groups were orally treated with aqueous extracts of *A. crispus* and *A. rigidifolius* at 90 mg/kg b.w. daily doses for 90 consecutive days. After 90 days, the same biochemical parameters mentioned above were evaluated along with hematological parameters using the rat serum. Then, the animals were sacrificed, and relative organ weights were determined.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) where necessary. The data obtained from each experiment were analyzed in Microsoft Excel 2016 and SPSS version 25 software. Analysis of variance (ANOVA) was conducted at the significance levels of $p < 0.001$, $p < 0.01$, and $p < 0.05$ using Bonferroni and Tukey post hoc tests.

RESULTS AND DISCUSSION

Pharmacognostic evaluation

Macroscopic assessment

This study on *A. crispus* and *A. rigidifolius* revealed that both plants lack floating leaves. Mature plants have single or several inflorescences, whereas younger plants do not have any inflorescence. The main distinctive character is their presentation of rootstock, i.e., *A. crispus* has a non-branching tuber, whereas *A. rigidifolius* has a branched rhizome. In addition to that, *A. crispus* has dense flowers, and *A. rigidifolius* has a slightly dense flower pattern. The flowers of both species are white with two tepals. Further, these flowers contain three ovaries and six stamens.

Microscopic anatomy

According to the microscopic assessment of different parts of *A. crispus* and *A. rigidifolius*, monocot-type vascular bundles were found in the petiole of both plants. The basal part of *A. crispus* leaf is triangular in a cross-sectional view. Towards the inner side of the epidermal layer, two to three layers of compact parenchyma cells were observed (Figure 1).

Powder microscopy

The powder microscopy of the petiole of *A. crispus* and *A. rigidifolius* resulted in fragments of large vessels, parts of epidermal cell layers, calcium carbonate crystals, and sclereids in both plants. Furthermore, the powder microscopy of *A. rigidifolius* petioles presented a striking image of the parenchymal cell layer, the four-cell thickness of the epidermal cell layer, fragments of large vessels with pitted zones, idioblast crystals, and sclereids (Figure 2).

Physicochemical properties

In the physicochemical assessment of plant materials, it was observed that *A. crispus* had slightly higher ash values compared to *A. rigidifolius*. In comparison, the acid-soluble ash content in *A. crispus* was found to be about two-fold

greater than in *A. rigidifolius*. Although fresh materials of both plants had almost similar moisture contents, powdered *A. crispus* showed a greater moisture level compared to powdered *A. rigidifolius* sample (Table 1).

Phytochemical analysis

Qualitative phytochemical screening

According to the results of the preliminary phytochemical screening of *A. crispus* and *A. rigidifolius*, cardiac glycosides were not present in any of the test extracts. Aqueous extracts of both plants yielded positive results for higher levels of alkaloids, phenols, flavonoids, tannins, proteins, and sugars. The dichloromethane extract of *A. rigidifolius* was positive only for a few phytochemicals, including phenolics and leucoanthocyanins (Table 2).

Table 1. Physicochemical properties of *Aponogeton crispus* and *A. rigidifolius*

Physicochemical properties	Plant sample (%)	
	<i>A. crispus</i>	<i>A. rigidifolius</i>
Weight loss on drying		
Moisture content of fresh sample	95.10±0.04	94.55±0.12
Moisture content of powdered sample	12.83±0.03	7.52±0.05
Ash values		
Total ash	18.74±0.05	17.69±0.03
Water soluble ash	7.16±0.28	6.51±0.22
Acid soluble ash	7.19±0.08	3.76±0.10
Extractive values		
Water	20.48±0.31	20.66±0.18
Ethanol	5.30±0.08	5.48±0.11
Dichloromethane	2.49±0.11	1.75±0.05

Note: Data values are expressed as mean of percentages ± SEM (n=3)

Table 2. Qualitative phytochemical screening of *Aponogeton crispus* and *A. rigidifolius*

Secondary metabolites	<i>A. crispus</i>			<i>A. rigidifolius</i>		
	Water	Ethanol	DCM	Water	Ethanol	DCM
Alkaloids	+++	++	+	++	+	-
Phenols	+++	++	+	+++	++	+
Flavonoids	++	+++	+	++	-	-
Tannins	+++	+	-	+++	-	-
Cardiac glycosides	-	-	-	-	-	-
Steroids	-	+	+	-	-	-
Terpenoids	+	-	-	++	-	-
Saponins	-	+	-	-	+	-
Leucoanthocyanins	-	++	+	+	-	+
Reducing sugar	+++	++	-	++	-	-
Protein and amino acids	++	++	+	+++	+	-
Coumarins	-	+++	+	++	-	-
Quinones	++	-	-	+	-	-

Note: DCM: Dichloromethane, '-': not detected, '+': trace, '++': moderate, '+++': appreciable amount

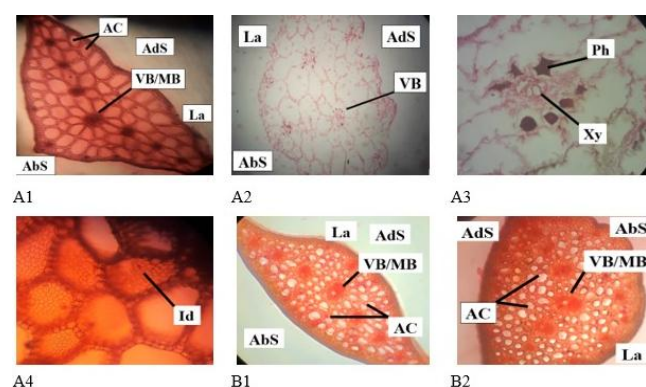


Figure 1. Microscopic view of transverse sections of A. *Aponogeton crispus*; B. *A. rigidifolius*, showing 1. Leaf, 2. Petiole, 3. Vascular system of the midrib, 4. Petiole with idioblasts. AbS: abaxial side, AdS: adaxial side, La: lamina, VB: vascular bundle, MB: median bundle, AC: aerenchyma cells, Ph: phloem, Xy: xylem, Id: idioblasts

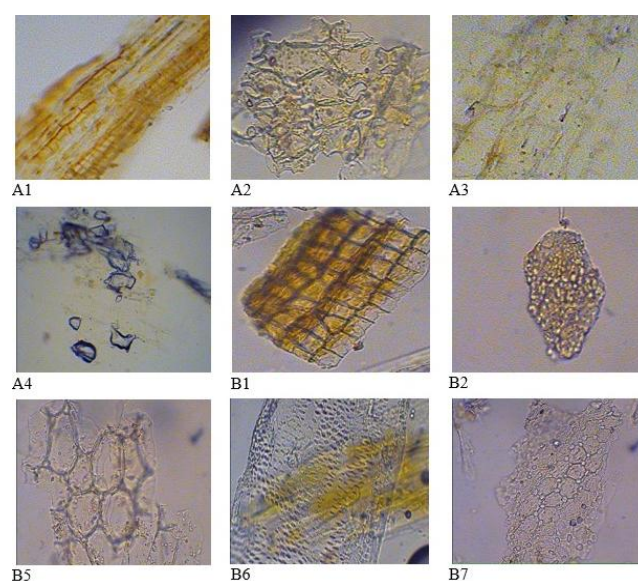
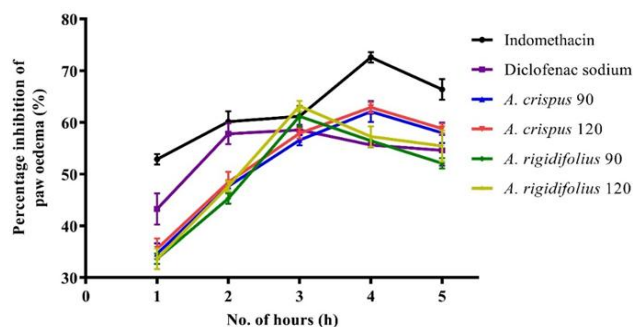
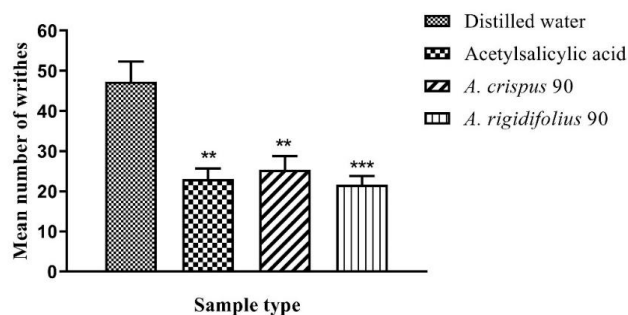


Figure 2. Photomicrographs of the plant powders of A. *Aponogeton crispus*; B. *A. rigidifolius*, showing 1. Epidermal cells, 2. Sclereids, 3. Fragments of large vessels, 4. Calcium carbonate crystals, 5. Parenchymal cells, 6. A fragment of large vessels with pitted zones, 7. idioblast crystals. 10×10

Table 3. Effect of aqueous extracts of *Aponogeton crispus*, *A. rigidifolius*, and acetylsalicylic acid on yeast-induced pyrexia

Test group	Rectal temperature after 18 hours of yeast injection (°C)	Rectal temperature (°C)			
		1 h	2 h	3 h	4 h
Distilled water	38.41±0.05	38.27±0.05	38.37±0.07	38.32±0.05	38.25±0.06
Acetylsalicylic acid 100 mg/kg	38.24±0.12	37.96±0.23	37.61±0.16*	37.38±0.12**	37.21±0.09***
<i>A. crispus</i> 90 mg/kg	38.39±0.12	38.29±0.16	38.16±0.12	37.53±0.15*	37.74±0.10*
<i>A. rigidifolius</i> 90 mg/kg	38.39±0.09	38.24±0.10	38.02±0.09	37.69±0.11*	37.32±0.09***

Note: Data values are expressed as mean ± SEM (n=6). Significant *p<0.05, **p<0.01, ***p<0.001 as compared to the control; ANOVA followed by Tukey's post-hoc test

**Figure 3.** Effect of aqueous extracts of both *Aponogeton crispus* and *A. rigidifolius* (at 90 and 120 mg/kg b.w.), diclofenac sodium, and indomethacin on carrageenan-induced paw edema; data values are expressed as percentage mean ± SEM (n=6), all the data are significant (p<0.05) when compared to the distilled water control; ANOVA followed by Tukey's post-hoc test**Figure 4.** Effect of aqueous extracts of both *Aponogeton crispus* and *A. rigidifolius* (at 90 mg/kg b.w.), and acetylsalicylic acid on number of writhes produced; data values are expressed as mean ± SEM (n=6). Significant *p<0.05, **p<0.01, ***p<0.001 as compared to the control; ANOVA followed by Tukey's post-hoc test

Quantitative determination of total phenolic content

Crude aqueous extracts of *A. crispus* and *A. rigidifolius* contain total phenolics of 72.69±1.87 mg GAE/g and 49.25±1.81 mg GAE/g, respectively.

Pharmacological studies

In vivo anti-inflammatory activity

As per the results of the carrageenan-induced hind paw edema assay, the maximum inhibition of edema by *A. crispus* was observed at the 4th hour with 90 mg/kg (62.09±2.21%) and 120 mg/kg doses (62.90±3.45%) following oral administration, whereas 61.18±3.42% and 63.15±5.26% of inhibitions were observed at the 3rd hour with the same doses of *A. rigidifolius*, respectively. Furthermore, different doses of the same test plant demonstrated similar patterns in anti-edemic activity interestingly (Figure 3).

In vivo antipyretic activity

Subcutaneous injection of yeast caused elevated rectal temperatures (38.24-38.41°C) in all rats used in this study. None of the test plant extracts were able to exhibit significant antipyretic activities (p>0.05) during the first 2 hours of the dose administration; however, both plant extracts (90 mg/kg) demonstrated significant body temperature reduction effects (p<0.05) during the 3rd and 4th hours (Table 3).

In vivo anti-nociceptive activity

Aqueous extracts of *A. crispus* and *A. rigidifolius* showed 25.33±3.50 and 21.67±2.10 mean number of

writhes, respectively, where the mean percentage reductions of writhes were 46.48% (p<0.01) and 54.22% (p<0.001) in the respective test group. Moreover, acetylsalicylic acid resulted in a 23.00±2.71 mean number of writhes (Figure 4).

In vitro thrombolytic activity

According to the percentage of clot lysis demonstrated by test groups, *A. crispus* dose of 135 mg/kg exhibited the highest activity (27.96±3.37%), which was comparable (p<0.05) to the standard drug, streptokinase (32.14±2.46%). Meanwhile, 180 mg/kg dose of the same extract demonstrated the lowest activity. *Aponogeton rigidifolius* doses of 90 mg/kg and 135 mg/kg demonstrated >20% of thrombolytic activity; however, it did not exhibit a significant effect (p>0.05) compared to the control. The statistical comparison confirmed that a negligible amount of clot lysis occurred with distilled water (Figure 5).

In vivo hypoglycemic activity

The aqueous extracts of *A. crispus* at the doses of 90 mg/kg and 180 mg/kg significantly (p<0.05) reduced the serum glucose levels in normal healthy rats. The postprandial serum glucose levels of the respective test groups were 3.64±0.35 and 3.87±0.32 mmol/L after 90 minutes of glucose loading. Among the four doses evaluated, the dose of 90 mg/kg appeared to be the most effective in reducing postprandial serum glucose levels in test animals (Figure 6.A). According to the serum glucose reduction determined in healthy Wistar rats following oral administration of 90 mg/kg dose of *A. crispus* against time,

the minimum serum glucose level of 3.64 ± 0.35 mmol/L was observed at 90 minutes from the administration of the test substance (Figure 6.B).

Toxicity studies

All of the test rat groups survived after being treated with a single dose of 2,000 mg/kg of aqueous extracts of *A. crispus*, *A. rigidifolius*, and distilled water for 14 days in the acute toxicity study. The animals showed no significant changes ($p > 0.05$) in their serum biochemical parameters. The results of histomorphological studies done at 14 days (as presented in Figure 7) also manifest no significant variations in the histology of heart, kidney, and liver tissues of the animals of the treatment group after the acute toxicity study.

All the animals tested in the sub-chronic toxicity study also survived without any significant abnormalities in tested serum biochemical or hematological parameters ($p > 0.05$). All rats gained weight through the study but this observation was comparable with the respective control groups. The relative organ weight percentage of the animals used in the sub-chronic toxicity assay (as indicated

in Table 4) did not show any significant alterations in test groups at any time point compared to the respective control group ($p > 0.05$).

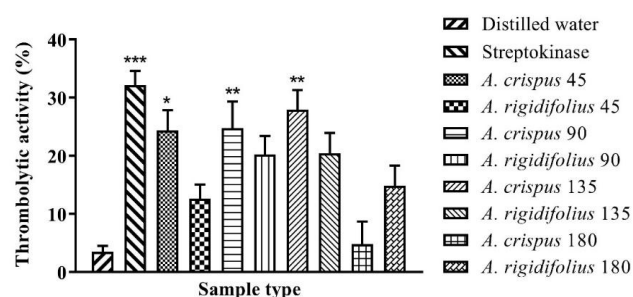


Figure 5. Effect of aqueous extracts of both *Aponogeton crispus*, *A. rigidifolius* (at 45, 90, 135, 180 mg/mL), and streptokinase on percentage clot lysis; data values are expressed as mean $\pm$ SEM (n=4). Significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to the control; ANOVA followed by Tukey's post-hoc test

Table 4. The relative organ weight percentage of Wistar rats after receiving a single dose of test substances for 14 days and consecutive doses for 90 days

	Relative organ weight (%) at 14th day			Relative organ weight (%) at 90th day		
	<i>A. crispus</i>	<i>A. rigidifolius</i>	Distilled water	<i>A. crispus</i>	<i>A. rigidifolius</i>	Distilled water
Liver	3.73 ± 0.09	3.95 ± 0.04	3.76 ± 0.06	4.28 ± 0.03	4.25 ± 0.04	4.26 ± 0.05
Kidney	0.46 ± 0.01	0.48 ± 0.01	0.44 ± 0.02	0.55 ± 0.01	0.56 ± 0.01	0.54 ± 0.02
Heart	0.49 ± 0.01	0.47 ± 0.01	0.49 ± 0.01	0.43 ± 0.01	0.43 ± 0.01	0.42 ± 0.01

Note: Data values are expressed as mean $\pm$ SEM (n=10). ANOVA followed by Tukey's post-hoc test

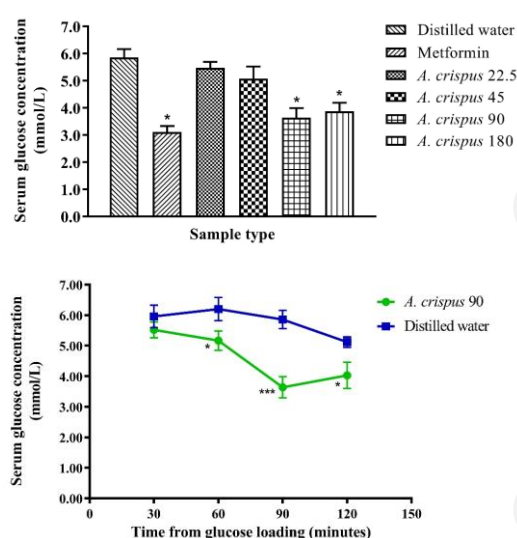


Figure 6. A. Effect of different doses of aqueous extract of *Aponogeton crispus* (at 22.5, 45, 90, 180 mg/kg b.w.) on serum glucose levels in healthy Wistar rats; B. Serum glucose reduction in healthy Wistar rats following oral administration of *A. crispus* aqueous extracts (90 mg/kg b.w.) with time; data values are expressed as mean $\pm$ SEM (n=6). Significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to the control; ANOVA followed by Bonferroni's post-hoc test

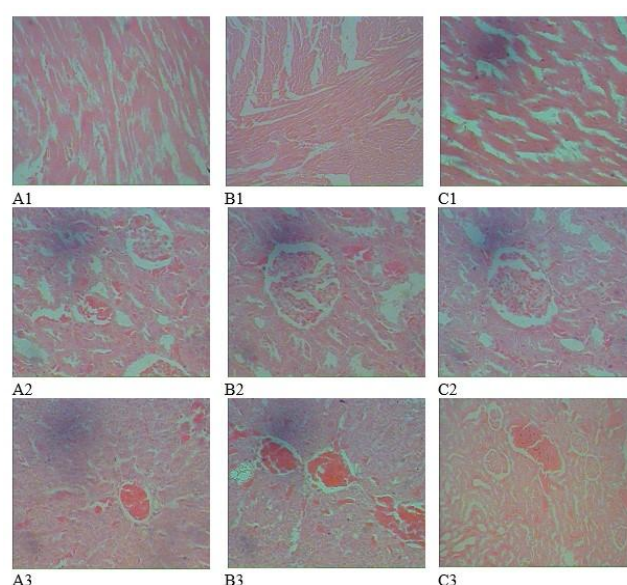


Figure 7. Effects of different *Aponogeton* plant extracts on histomorphology of various rat organs in acute oral toxicity study. A. Control group; B. Group treated with *A. Crispus*; C. Group treated with *A. rigidifolius*. 1. Heart tissues, 2. Kidney tissues, 3. Liver tissues. All the sections were hematoxylin and eosin (H & E) stained. 10×10

Discussion

As observed in our study, the morphology of both *A. crispus* and *A. rigidifolius* aligns with the initial monographs provided by Dassanayake and Fosberg in general (Dassanayake and Fosberg 1987). However, it was also observed that the length of the studied *Aponogeton* leaves vary in mature plants, depending on the depth of the habitat water. In pharmacognostic analysis, both of the plants yielded comparable levels of extractable matter, and the extractable contents in more polar solvents were found to be higher. This observation is supported by another study done on *A. natans*, where higher levels of extractable matter were observed in more polar solvents, compared to non-polar solvents (Dash et al. 2015). The absence of cardiac glycosides reported in the present phytochemical screening has also been observed in other *Aponogeton* species, making it a unique characteristic of *Aponogeton* genus (Nandagoapalan et al. 2016). In a separate study, the ethanolic extract of *A. natans* had resulted in a higher content of total phenolics compared to the less polar chloroform extracts. High phenolic contents in these test plants are critical since a number of bioactivities are thought to be mediated through the free-radical scavenging action of phenolic compounds (Janarny et al. 2021). Variation in phytochemical composition due to internal and external botanical factors influences the biological activities of the plant materials. Since the present study furnishes only preliminary evidence for medicinal use of *Aponogeton* species, further studies should incorporate samples from diverse geographical locations, environmental conditions, and herbal varieties to assess the impact of these variables in phytochemistry and pharmacological activity (Biondi et al. 2021).

Ethnopharmacology often bridges traditional uses and scientific usage of medicinal plants. Research has shown that many plants used in traditional medical systems possess pharmacological properties that can be scientifically validated by established methodologies (Xing et al. 2021). The study of different doses of the same *Aponogeton* species showed no significant changes in the pattern of anti-inflammatory effect in the carrageenan-induced paw edema assay, other than dose-dependent variation. Carrageenan can release inflammatory and pro-inflammatory mediators such as histamine, prostaglandins, TNF- α , and bradykinin. Much evidence has been brought forward to prove that polar phytochemicals such as phenolics, triterpenoids, and flavonoids possess anti-inflammatory and antipyretic activities, especially by their interaction with the above-mentioned inflammatory mediators (Mansouri et al. 2015). Therefore, the present study suggests that the presence of such phytochemicals might be the underlying cause of the anti-inflammatory activity produced by *A. crispus* and *A. rigidifolius*. In a similar paw edema study, methanolic *A. natans* extract had showed a significant ($p < 0.01$) inhibition of edema at the 4th and 5th hours of the experiment. In contrast, the petroleum-ether and benzene extracts of the plant had showed lesser activity (Dash et al. 2013), which justifies the greater anti-inflammatory activity demonstrated by the aqueous extracts

of *A. crispus* and *A. rigidifolius* in the present rat paw edema model.

Studies have suggested that the antipyretic properties in plant extracts are likely linked with the interference of eicosanoids biosynthesis and inhibition of neutrophil degranulation in animals. These actions, when combined, result in the suppression of inflammatory mediators such as prostaglandins and lipoxygenase (Roe 2021). Furthermore, the sequence and pattern of body temperature lowering by test plants were almost similar to the standard drug, establishing that the antipyretic property of these plants may be mediated through the inhibition of prostaglandin synthesis similar to acetylsalicylic acid. The acetic acid-induced writhing test is recognized as a common in vivo method to evaluate compounds with peripheral analgesic effects. The injection of acetic acid sensitizes the analgesic receptors to prostaglandins or endogenous opioids (Rege et al. 2021). Therefore, it is suggested that the herbal extracts tested in the present study may provide analgesic activity by antagonizing the activity of prostaglandins and/or other inflammatory mediators in the body.

The thrombolytic activity of any substance is associated with the disruption of blood clots formed in an inflammatory condition. A previous study carried out on methanolic extract of *A. undulatus* has shown moderate thrombolytic activities (Chowdhury et al. 2011). According to the same study, saponins, alkaloids, and tannins were presumed to be responsible for the thrombolytic activity exerted by the plant. Especially, saponins have been repeatedly proven for their hemolytic activity through the disruption of blood cell membranes (Bissinger et al. 2014). Since the same type of phytochemicals was found in both of the test plants of the present study, it could be highlighted as the most probable reason for the in vitro thrombolytic activity. Apart from the biological effects of *A. crispus* and *A. rigidifolius* against the classical signs of inflammation, an appreciable anti-hyperglycemic activity was further demonstrated by *A. crispus* aqueous extract. Chloroform extracts of *A. natans* have also reported a significant ($p < 0.05$) anti-hyperglycemic activity in a previous study (Dash et al. 2014).

Toxicity studies are essential for herbal materials with potential for drug development (Chen et al. 2022). The absence of statistically significant differences in toxicity parameters between the test and control animal groups of the present study justifies the use of *Aponogeton* spp. as a traditional food in different South Asian ethnic groups (Jayaweera and Senaratna 2006; Misra et al. 2012). Previous studies suggest that *A. crispus* and *A. rigidifolius* contain various phytochemicals, including quercetin, gallic acid, cyanidin, stigmasterol, p-hydroxybenzoic acid which are known for their anti-inflammatory and anti-hyperglycemic properties (Abdelkhalek et al. 2024). Specially, no specific phytochemical compound has been isolated or characterized from *A. rigidifolius* so far in any reported study. Isolation and characterization of compounds in a future study will provide solid evidence on its medicinal role. Results of the present study urge the need for an extensive study to demonstrate the molecular mechanisms in aqueous extracts of *A. crispus* and *A.*

rigidifolius with respect to their in vivo and in vitro biological activities evidenced here.

It is concluded by this study that the aqueous extract of both *A. crispus* and *A. rigidifolius* demonstrated significant acute anti-inflammatory activity by acting against the key signs of the inflammation, i.e., edema, pain, fever, and thrombosis. Interestingly, *A. rigidifolius*, which is native to Sri Lanka exerts higher analgesic and antipyretic effects in comparison to *A. crispus*. The hypoglycemic effect of *A. crispus* was greatest at 90 minutes, and a time-dependent activity was also evident. Moreover, both plants were found to be safe for consumption in acute or chronic settings, as per the findings of the toxicity study; therefore, they could be recommended as functional foods. The pharmacognostic findings of this study could be used as standard parameters to differentiate and standardize *A. crispus* and *A. rigidifolius* in ethnomedicine.

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Characterization of caffeinated facial creams with *Diploknema butyracea* butter as an emollient

SAPANA SUBEDI, SANTOSH KOIRALA, PRAYAN POKHAREL, DHAKA RAM BHANDARI*

Gandaki Province Academy of Science and Technology (GPAST), Pokhara 7, 33700, Nepal.
Tel.: +977-9856086486, *email: director.gpast@gandaki.gov.np

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Abstract. Subedi S, Koirala S, Pokharel P, Bhandari DR. 2025. Characterization of caffeinated facial creams with *Diploknema butyracea* butter as an emollient. *Asian J Nat Prod Biochem* 23: 11-18. Caffeine, a versatile ingredient in cosmetics, is known for its vasoconstrictive, anti-inflammatory, UV protective, and anti-aging properties. The plant *Diploknema butyracea*, a medium-sized tree belonging to the Sapotaceae family, native to Nepal, India, and Bhutan, is known as *chyuri*, and its seeds contain about 55% fat, known as *chyuri* butter. *Chyuri* butter is rich in polyunsaturated fatty acids and vitamins (A and E) to hydrate, nourish, soften skin and serve as an effective emollient. In this study, we formulated caffeinated facial creams with *D. butyracea* butter and meticulously evaluated their physical and chemical properties. Caffeine was extracted via liquid-liquid extraction. Four formulations (F1 to F4) with varying concentrations of *chyuri* butter and caffeine were assessed for pH, stability, acid and saponification values, spreadability, in-vitro occlusivity, centrifugation, after-feel, smear type, removal, and dilution. The pH of the creams ranged from 6.7 to 7.6. All formulations, except F1, remained stable and homogeneous at different temperatures. Acid values (9.5-11.2) and saponification values (23.5-26.8) were satisfactory. The creams demonstrated good spreadability (4.3-4.5 cm) and improved water retention due to *chyuri* butter. No phase separation occurred during centrifugation. The creams formed a non-greasy film, were easily removable with tap water, and were confirmed as O/W (oil in water) type. Our findings not only validate the efficacy of caffeine and *chyuri* butter in cosmetic cream formulations but also open up new possibilities for their use in skincare products.

Keywords: Caffeine, *chyuri*, cosmetics, *Diploknema butyracea*, facial cream

INTRODUCTION

Throughout history, different natural and synthetic ingredients have been used for beauty. Due to the presence of secondary metabolites, various compounds of herbal plants possess antimicrobial, anticancer, anti-inflammatory, and other pharmacological properties (Larbie et al. 2019; Katyal 2022). Natural ingredients and their potent compounds are gaining popularity in cosmetic products. Cosmeceutical products containing natural ingredients provide the skin with essential nutrients such as vitamins A, C, and E, phenolic compounds, flavonoids, and terpenoids. These components act as antioxidants and anti-inflammatory agents and have anti-aging and skin-brightening effects, ultimately promoting and improving skin health (Lohani et al. 2019). Cosmetic products are extensively used to safeguard the skin from harmful external and internal elements to improve the skin's appearance and beauty (Kaur and Saraf 2010; Liu 2022). Cosmeceuticals enhance external attractiveness and promote longevity by mitigating skin disorders (Datta and Paramesh 2010; Kumar et al. 2024).

Caffeine (1, 3, 7-Trimethylpurine-2,6-dione), an alkaloid found in coffee and tea, is a central nervous system stimulant. Caffeine is widespread in the cosmetics industry due to its potent biological activity and ability to penetrate the skin barrier (Blanco-Llamero et al. 2024). It exhibits significant antioxidant and anti-aging activities,

shielding cells from UV radiation and decelerating the skin's photoaging process (Herman and Herman 2012). Caffeine also possesses anticellulite properties by inhibiting adrenergic receptors and hindering excessive fat buildup through accelerated lipolysis. The impact of a caffeine-containing emulsion on adipose cells was investigated by topically applying it to Wistar female mice for 21 days, resulting in a 17% decrease in adipose cell diameter compared to the control group (Velasco et al. 2008). Furthermore, caffeine has been proven to increase vasoconstriction, tighten the skin, and reduce swelling (Lee et al. 2019), providing a solid foundation for its use in cosmetic products and instilling confidence in its effectiveness.

According to the FDA, animal testing is not specifically required for marketing a cosmetic product; however, any testing relied upon should be scientifically sound. FDA regulations state that cosmetic products and ingredients, except for color additives, do not require FDA approval before being introduced. However, they must not be "adulterated" or "misbranded." When applied near the eyes, lotions containing caffeine and theobromine are limited to maximum concentrations of 1.5 and 0.0025%, respectively (Cherian 2018).

Emollient bases improve skin moisture and flexibility by forming a protective layer on the skin's surface. This layer helps prevent water loss from the underlying skin layers (occlusive effect) and traps moisture from the surrounding environment (moisturizing effect) (Stojiljković

et al. 2013). Despite the growing popularity of cosmetic creams, several challenges have emerged, including the instability of the chemical base, the need for multiple excipients to ensure stability, the high production costs of synthetic bases, and the potential for the base to cause skin hypersensitivity reactions. These challenges have driven the investigation into more effective emollients from natural sources (Pandey et al. 2021).

Diploknema butyracea plant, a medium-sized tree belonging to the family Sapotaceae, is native to Nepal, India, and Bhutan and found at altitudes ranging from 300 to 1,500 meters above sea level. It grows mainly in the sub-Himalayan tracts on steep slopes and cliffs. Known as "chyuri" in Nepal and "Indian-butter nut" in English, its primary product is "ghee" or butter extracted from the seeds, named "chiuri ghee." The *chyuri* seed contains approximately 55% fat, known as *chyuri* butter, serving as a vegetable oil and massage oil for conditions such as rheumatism, headache, acne, and boils. *Chyuri* fat is a valuable natural emollient, particularly during winter, for moisturizing and nourishing cracked feet and hands (Anand et al. 2024). Furthermore, numerous commercial enterprises have experimented with developing various products such as chyuri syrup, chyuri jam, and chyuri cream (Pandey et al. 2021).

Among various topical preparations in cosmeceuticals, creams are semisolid uniform preparations intended for use on the skin's surface or external mucous membranes. In this study, caffeinated facial cream, including *D. butyracea* butter as an emollient, was formulated and evaluated for physical and chemical parameters. The study aimed to develop cosmetic creams using locally available herbal ingredients with a novel approach. Instead of using whole plant extracts, the formulation utilized a single potent compound of the plant as an active ingredient.

MATERIALS AND METHODS

Chemical and ingredients

Table 1 contains a list of the chemicals and ingredients used in the experiments, along with their corresponding brand names and suppliers.

Extraction of caffeine

We extracted caffeine from coffee using the method by Mumin et al. (2006) with slight modifications. Coffee beans were locally purchased from Pokhara Metropolitan, Nepal, and were grounded into fine powder particles using a mixer grinder. A 10% w/v solution of powdered coffee was prepared using acidic water (pH adjusted to 2 using hydrochloric acid). The mixture was boiled for 20 min at 100°C while stirring and then filtered. Sodium carbonate was added to the filtrate to make the solution basic (pH 10). Next, a 500 mL separatory funnel was placed on an iron ring attached to a ring stand. The basic filtrate solution was poured into the separatory funnel, adding approximately 10 mL of dichloromethane. The solution was thoroughly shaken, and the stopcock was opened to release any vapors. After separating the layers, the lower layer (dichloromethane) was carefully collected into a 100 mL beaker. This separation process was repeated three times, using 10 mL of dichloromethane each time, and all the collected dichloromethane layers were combined in the beaker. Dichloromethane was evaporated using a rotary evaporator. The temperature was kept at a controlled low level, ranging from 30 to 35°C. As a result of the dichloromethane evaporation, crude caffeine solidified at the bottom of the beaker. Finally, to obtain pure caffeine crystals, 10 mL ethanol was used for recrystallization.

Measurement of the extraction yield

The weight of pure caffeine crystals obtained was measured, and the yield percentage was calculated using the following formula:

Extraction yield (%) = [Weight of caffeine crystals obtained/Weight of coffee powder taken] × 100

Table 1. Chemicals and ingredients used in this study with their brand and supplier

Chemicals and ingredients	Brand	Supplier
Coffee beans	Whole bean/Arabica Coffee	Machhapuchhre Organic Coffee
Hydrochloric acid (Chlorane)	Paskem	Paskem Fine Chem Pvt. Ltd.
Sodium carbonate	Himedia	Himedia Laboratories Pvt. Ltd.
Dichloromethane	CDH	Central Drug House Pvt. Ltd.
Ethanol	-	Changshu Hongsheng Fine Chemical Pvt. Ltd.
Stearic acid (octadecanoic acid)	Nike	Nike Chemical India
Cetostearyl alcohol	Loba Chemie	Loba Chemie Pvt. Ltd.
Triethanolamine (2,2',2''-Nitrilotri(ethan-1-ol))	Alpha Chemika	Alpha Chemika
Glycerol propan-1,2,3-triol	Qualigens	Thermo Fisher Scientific India Pvt. Ltd.
Propylparaben (Propyl 4-hydroxybenzoate)	Loba	Loba Chemie Pvt. Ltd.
Chyuri butter	Himalayan bio	Daraz, Nepal
Ether (Ethoxyethane)	CDH	Central Drug House Pvt. Ltd.
Phenolphthalein 3,3-bis(4-hydroxyphenyl)-2-benzofuran-1(3H)-one ()	Qualigens	Thermo Fisher Scientific India Pvt. Ltd.
Potassium hydroxide	Qualigens	Thermo Fisher Scientific India Pvt. Ltd.

Table 2. Concentration (% w/w) of ingredients used for four cream formulations and their uses

Ingredients	F1	F2	F3	F4	Uses
Stearic acid	5	5	5	5	Emollient, emulsifier, and stabilizer
Cetostearyl alcohol	3.75	3.75	3.75	3.75	Emulsifier
Triethanolamine	0.62	0.62	0.62	0.62	pH adjuster and emulsifier
Glycerol	2.5	2.5	2.5	2.5	Humectant
Propyl paraben	0.04	0.04	0.04	0.04	Preservative
Chyuri butter	0	20	20	20	Emollient
Caffeine	0	0	0.5	1	Active ingredient
Distilled water	88.09	68.09	67.59	67.09	Solvent

Cream formulation

The cream was formulated using the method by Farboud et al. (2011) with slight modifications. The necessary quantity of the aqueous and lipid phases was individually heated to $70\pm 2^\circ\text{C}$. The aqueous phase was then carefully added to the lipid phase while continuously stirring with a hand blender until it solidified. Four different formulations were developed, each containing 0% (F2), 0.5% (F3), and 1% (F4) caffeine, with varying amounts of chyuri butter serving as the emollient and control with 0% caffeine and 0% chyuri butter (F1). These formulations, with their specific caffeine and chyuri butter concentrations, were subjected to various evaluations. The components employed in the cream formulation are provided in Table 2. According to the FDA, ingredients used in cosmeceuticals should be safe per the safety data provided by Cosmetic Ingredient Review (CIR). All the ingredients listed are safe per the safety guidelines available on the CIR website (CIR 2016).

Cream formulation

Formulated creams were stored at three distinct temperature settings: $8\pm 2^\circ\text{C}$, $25\pm 2^\circ\text{C}$, and $40\pm 2^\circ\text{C}$. A stability study was conducted on the 0th, 7th, 14th, and 21st days (Smaoui et al. 2017). The cream (0.5 g) was carefully weighed and dissolved in 50 mL of distilled water. Subsequently, the pH of the solution was determined by employing a pH meter. The color and homogeneity of formulations were judged through visual observation and touch (Saheb et al. 2018).

Acid value

The acid values of creams were assessed using the method outlined by Saraf et al. (2010). Next, 5 g of cream was dissolved in a 50 mL equimolar mixture of ethanol (95%) and ether. The flask was attached to a reflux condenser and heated slowly until the cream dissolved. In the mixture, 1 mL of phenolphthalein was introduced and titrated with 0.1 N NaOH until a faintly pink color was observed. The acid value was then calculated using the following formula:

$$\text{Acid value} = [N \times 5.61]/W$$

Where:

N: the number of mL of NaOH required

W: the weight of the substance.

Saponification value

The saponification values of creams were assessed using the method outlined by Aswal et al. (2013). Briefly, 2 g of the formulated cream was mixed with 25 mL of 0.5 N ethanolic KOH and refluxed for 30 minutes. After adding 1 mL of phenolphthalein indicator, the solution was titrated with 0.5 N HCl.

The saponification value (SV) was calculated by applying the following equation:

$$SV = [(b-a) \times 28.05]/W$$

Where:

a: the volume in mL of titrant

b: the volume in mL of the titrate

SV: the saponification value

W: the weight of the substance in g

Spreadability

To measure the spreadability of the test formulation, 0.5 g of the sample was placed within a 1 cm diameter circle marked on a glass plate. Then, it was covered with another glass plate, and a weight of 500 g was placed on the top glass plate for 5 minutes. Spreadability refers to the area covered by a fixed amount of the cream sample after evenly spreading it on the glass slide. The diameter increase caused by the test formulation's spreading was recorded (Maru and Lahoti 2018).

In vitro occlusivity test

The occlusivity of the formulations were determined using the method of Maru and Lahoti (2018). Beakers with a diameter of 3.2 cm and height of 4.6 cm were used for the occlusivity test. In each beaker, 10 g of distilled water was placed by covering the top with Whatman filter paper (with pores of size $0.45 \mu\text{m}$), and 200 mg of the sample was evenly spread on the filter paper. After that, these beakers were kept at a constant temperature of $37\pm 2^\circ\text{C}$ and relative humidity of $60\pm 5\%$ for 48 hours. All the formulations and negative control (where the filter paper was left uncovered) were examined to measure the amount of water vapor that passed through the samples and determine the occlusivity of the formulations.

The occlusion factor F was calculated as follows:

$$F = (A-B) / A \times 100$$

Where:

A: Water flux through an uncovered filter (percent water loss)

B: Water flux through the filter when covered by test preparation (percent water loss)

Centrifugation tests

Centrifugal tests were conducted on freshly prepared emulsions. The tests were performed at 5,000 rpm and 25°C for 10 minutes, with 10 g of each sample placed in separate centrifugal tubes (Smaoui et al. 2017).

After feel and type of smear

Emollience, type of smear and the quantity of residue after applying a fixed amount of cream were assessed using the method by Dhase et al. (2014). An area was marked on the dorsal side of the left hand with a marker. 0.5 g of the formulations were uniformly applied over the marked area spreading it gently to form an even layer. The treated area was examined for changes in texture, smoothness and softness which indicate the emollience. The type of smear was categorized as greasy and non-greasy reflecting the absorbability of the formulations. The quantity of residue was measured qualitatively by lightly wiping the area with a clean towel and observing the transfer.

Removal

The ease of removal of the cream applied to the treated area was evaluated by rinsing it with tap water after half an hour of application (Mishra et al. 2014). The treated area was subjected to a gentle stream of tap water at ambient temperature. After rinsing, the treated area was patted dry with a clean towel, and the skin was examined for any lingering residue, greasiness, or film.

Dilution test

The dilution test was conducted to identify the emulsion type of the cream, either oil-in-water (O/W) or water-in-oil (W/O). 1 g of the cream formulation was placed into each of two beakers. In the first beaker, 10 mL of distilled water

was gradually added while stirring. In the second beaker, 10 mL of oil was added under the same conditions. The mixtures were examined for any phase separation, or clumping. If the cream formulation mixed uniformly with water without separation, it was classified as an O/W emulsion. If the cream mixes uniformly with oil but not with water, it was classified as W/O emulsion (Dhase et al. 2014).

RESULTS AND DISCUSSION

Caffeine extraction and cream preparation

The extraction yield of caffeine crystals (shown in Figure 1.A) was 0.13%. Four formulations labelled F1, F2, F3 and F4 (shown in Figure 1.B) with varying concentrations of caffeine and chyuri butter (shown in Figure 1.C) were prepared. The concentrations of each ingredient are listed in Table 2. These formulations were evaluated for various parameters to assess their properties and performance.

Stability of creams

The ideal pH range for cosmetic creams should lie between 5 and 8 (Saraf et al. 2010). The formulations' pH was within the specified range of 6.7-7.6 (Figure 2). The pH of the formulations remained relatively constant during the study. Formulations containing chyuri butter (F2, F3, and F4) were white, while formulations not containing chyuri butter were transparent white (Figure 3). The appearance study of all the formulations showed homogeneity. Their appearance did not change at $8\pm 2^\circ\text{C}$, $25\pm 2^\circ\text{C}$, and $40\pm 2^\circ\text{C}$ except for formulation F1, which showed non-homogeneity with grittiness at $8\pm 2^\circ\text{C}$ (Figure 3). The centrifugation test relies on centrifugal force to separate substances of different densities. This test is used to evaluate and forecast the stability of emulsions (Khan et al. 2010). In this study, there was no phase separation of all the formulations on centrifugation (Figure 3).



Figure 1. A. Extracted caffeine crystals; and B. Prepared four formulations with concentrations of 0, 0.5, and 1% caffeine; C. Incorporating chyuri butter

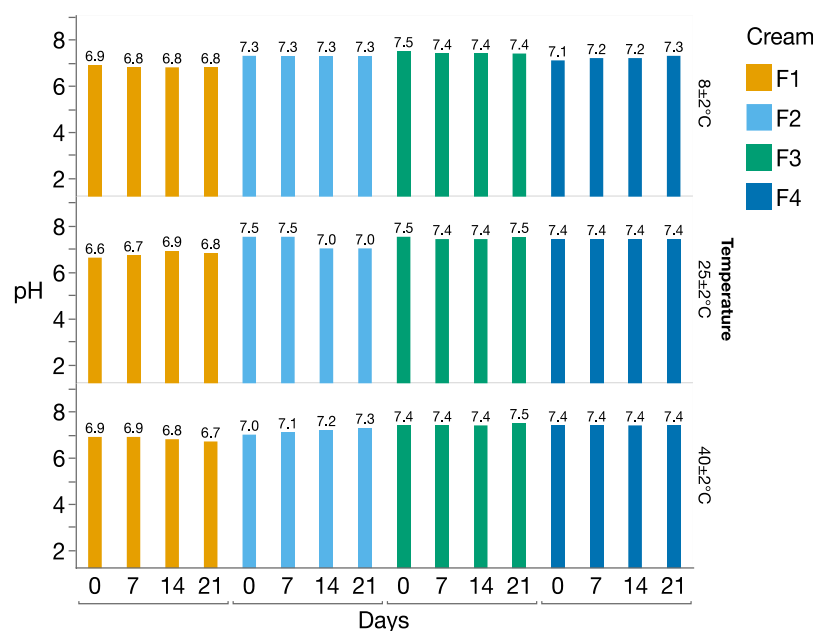


Figure 2. The pH range of the four formulations at three different temperatures ($8\pm 2^\circ\text{C}$, $25\pm 2^\circ\text{C}$, and $40\pm 2^\circ\text{C}$) in 0th, 7th, 14th, and 21st days

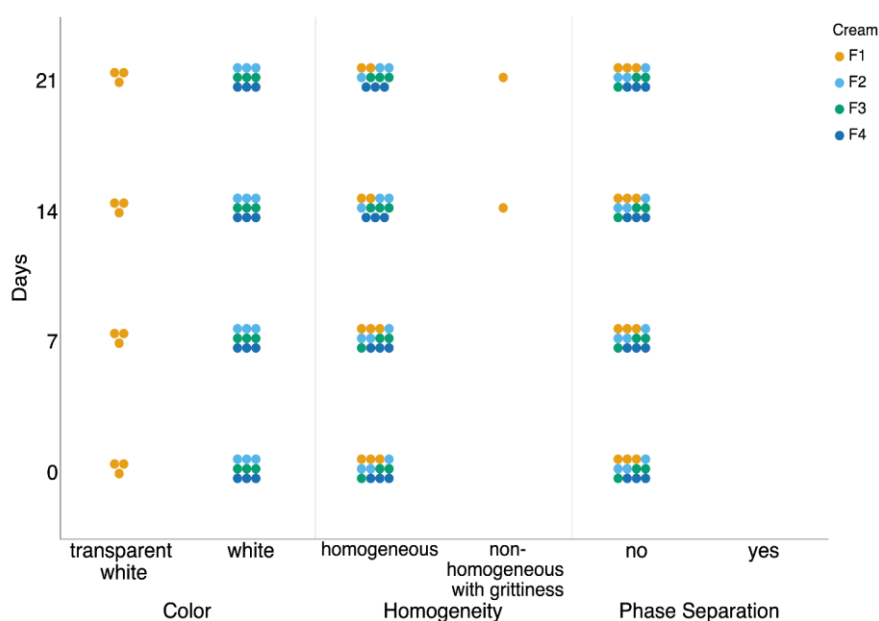


Figure 3. Color (left), homogeneity (middle) and phase separation (right) of the four formulations. Each point shows whether the formulations are transparent white or white (left), homogeneous or non-homogeneous with grittiness (middle) and phase separated (right)

Physical parameters

The acid and saponification values of all cream formulations were under the acceptable range (between 9.5-11.2 and 23.4-26.7, respectively) (Figure 4). This value indicates that the cream's stability would be higher, reducing the likelihood of degradation or rancidity (Panda and Mishra 2018). The spreadability of formulated creams were between 4.3-4.5 cm, which indicates good spreadability (Figure 4) (Maru and Lahoti 2018). The

higher the occlusivity value, the more water loss is prevented in a higher proportion from the system and vice versa. The rate of water loss from the skin (water flux) is influenced by the occlusive properties of the formulations applied to the skin. The cream's water-retaining properties were improved by adding *chyuri* butter, which was evident from the increased percentage of in-vitro occlusivity (Figure 4).

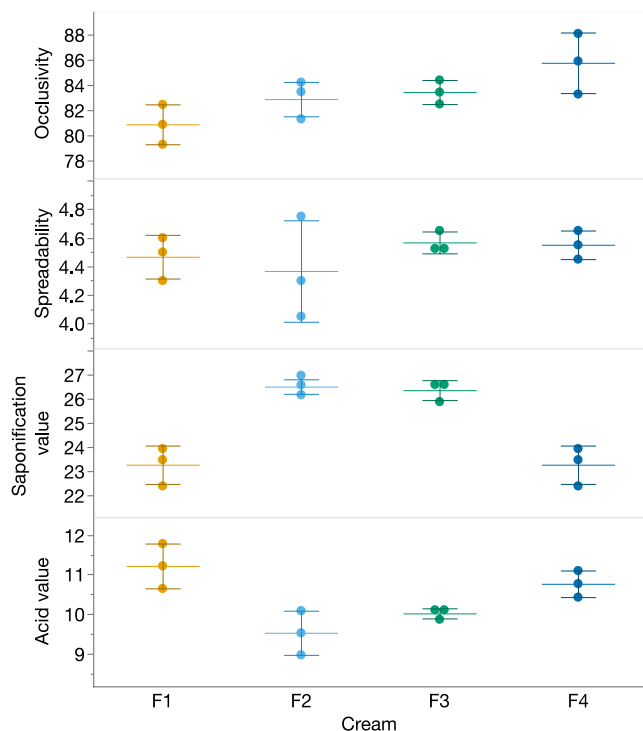


Figure 4. Acid value, saponification value, spreadability, and occlusivity of the four formulated creams. Each horizontal bar represents the mean ($\pm$ SD) of the physical parameters

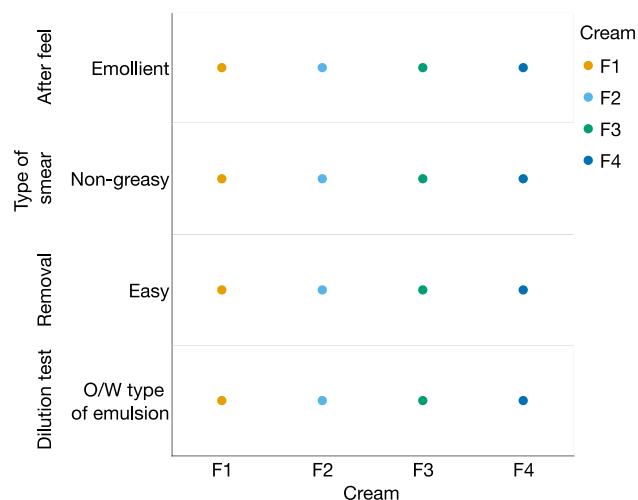


Figure 5. Each point represents parameters such as emulsion type, easy removal, greasiness, and emollience

Other parameters

The investigation revealed that the creams created a non-greasy layer on the skin's surface with emollient characteristics upon application. Moreover, no residue was left on the skin surface after applying, and removal was found easy under tap water. All of the formulated creams were found to be O/W (oil in water) type by dilution test (Figure 5).

Discussion

Nepal's involvement in commercial coffee cultivation is renowned in global markets for its unique flavor and aroma. Coffee is produced at elevated altitudes, distant from the primary coffee-growing Capricorn and Cancer Belt (beyond 23° latitude). Coffee beans from Nepal are primarily exported to countries like Japan, the United States, and various European nations with the trend now expanding to other regions. Caffeine, a significant component in coffee, offers a plethora of health benefits. Caffeine, which is a significant component of coffee, diminishes puffiness around the eyes, hastens the drainage of the lymph system from fatty tissue, enhances blood microcirculation in capillary vessels, has anti-cellulite properties, triggers lipolysis, and releases excess fat from adipocyte cells by decreasing their size (Herman and Herman 2012).

Chyuri butter is rich in polyunsaturated fatty acids and vitamins (A and E) that help hydrate, nourish, and soften our skin. Polyunsaturated fatty acids present in *chyuri* butter are palmitic acid (50-60%), oleic acid (30-40%), linoleic acid (2-10%), and stearic acid (0-5%) (Dahal et al. 2021). Due to these constituents, *chyuri* fat has a rich historical significance in economic and ethnomedicinal importance among various ethnic groups in Nepal. It has been widely employed as a key ingredient in numerous commercial formulations by cosmetic, pharmaceutical, and soap companies (Pandey et al. 2021).

The economic potential of *D. butyracea* (*chyuri*) butter in Nepal is significant due to its diverse uses, ranging from culinary and medicinal applications to cosmetics and industrial products. With a growing demand for natural products globally, *chyuri* butter could be a high-demanded product for international consumers. For example, between 2011 and 2016, approximately 20 metric tons of *chyuri* butter were exported annually from Nepal to the European market. Cosmetic items incorporating *chyuri* butter have already been introduced into the European market. Brands like Officina Naturae and Cime have already unveiled skincare products, utilizing *chyuri* butter as a fundamental ingredient for creams, ointments, and lotions. Cosmetic items incorporating *chyuri* butter have already been introduced into the European market. Brands such as Officina Naturae and Cime have already launched skincare products, utilizing *chyuri* butter as a base for creams, ointments, and lotions (Devkota et al. 2019). The production and commercialization of *chyuri* butter can create income opportunities for local communities, foster small-scale industries, promote value chain development, and enhance both rural and national economies, all while encouraging environmentally responsible practices and preserving traditional knowledge.

The properties mentioned earlier make caffeine and *chyuri* butter important biological compounds that can be used in different cosmetic products. The ingredients used in our formulation constitute both acidic and basic components. Stearic acid and propylparaben contribute to acidic pH, while cetostearyl alcohol, triethanolamine, glycerol, *chyuri* butter, and caffeine contribute to basic pH. Assessing the pH is essential for evaluating the stability of

pharmaceutical products and cosmeceuticals. Any changes in pH represent potential interactions and degradation, impacting the product's quality (Smaoui et al. 2017). Most cosmetic and pharmaceutical creams are formulated as either "Oil in Water" (O/W) or "Water in Oil" (W/O) emulsions. Oil in water emulsion is non-greasy and is easily removable from the skin surface.

In contrast, water in oil emulsion is greasy and not water-washable. Water-soluble medications are released more rapidly from oil-in-water emulsions (Barkat et al. 2011). Caffeine is water soluble; thus, the creams in our study are formulated as O/W emulsions. The instability of emulsion can be due to various factors such as phase inversion, creaming (or sedimentation), coalescence, or breaking (Masmoudi et al. 2005). This research subjected the formulations to different storage conditions, including 8, 25, and 40°C. The formulations were examined for changes in pH, color, and phase separation. The pH of samples was found to be acidic to slightly basic. The pH of the formulations remained relatively constant during the study. The prepared creams containing chyuri butter were white. Their appearance did not change at $8\pm 2^\circ\text{C}$, $25\pm 2^\circ\text{C}$, and $40\pm 2^\circ\text{C}$ except for formulation F1, which showed non-homogeneity with grittiness at $8\pm 2^\circ\text{C}$. The change in appearance in F1 appeared from the 14th day and persisted up to the 21st day of the analysis period. The alteration in the appearance of F1 throughout the observation period could likely be attributed to the separation of the oily phase, which was encouraged by low temperatures. Moreover, formulation F1 did not contain chyuri butter as an emollient, which suggests that *chyuri* butter increases the stability of the formulation.

This study found creams' acid and saponification values between 9.53-11.21 and 23.47-26.77, respectively (Figure 4). Cream with 1% caffeine exhibited an acid value of 10.77 ± 0.46 and a saponification value of 26.76 ± 0.46 (Figure 4). In a previous study, a caffeinated fairness cream with 1% caffeine was formulated, demonstrating an acid value of 5.9 and a saponification value of 25.7 (Panda and Mishra 2018). The disparity in acid value and saponification value could be attributed to variations in the ingredients utilized in the formulations. The saponification value of the formulation increases with a higher concentration of fatty acids, and the formulation has more chance of microbial degradation (Saraf et al. 2010). In our investigation, we discovered that the saponification value of the cream (Figure 4) increased with the addition of chyuri butter; therefore, creams containing chyuri butter were more prone to microbial growth than creams not containing *chyuri* butter.

Our study found that the in vitro occlusivity percentage of creams increased with adding *chyuri* butter. The improved occlusivity observed in creams containing chyuri butter can be due to the enhanced lipid content of the developed formulations, leading to more effective prevention of water evaporation (Maru and Lahoti 2018). Thus, *chyuri* butter can moisturize and soften the skin, which helps to provide a protective barrier on the skin's surface, helping to retain moisture and prevent dryness,

making the skin feel smoother. Thus, it can be used as an emollient on skin care products.

There was no phase separation of all the formulations on centrifugation, indicating that the emulsion forming the cream was stable (Figure 3). This could result from the appropriate homogenization speed during the emulsion formulation, which could have prevented the formulations from breaking on centrifugation (Nour and Yunus 2006). Hence, the findings revealed potential possibilities for utilizing caffeine and chyuri butter in cosmetics creams. The discovery of novel biological properties of caffeine is expected to expand caffeine utilization in the fields of cosmetology and dermatology in the future (Herman and Herman 2012). The study suggests that an O/W cream formulated with stearic acid, cetostearyl alcohol, triethanolamine, glycerol, propylparaben, distilled water, 20% chyuri butter, and 0.5-1% caffeine yields stable creams with good physical characteristics.

Future studies should focus on validating the chemical and physical stability of these formulations containing caffeine and chyuri butter at different climatic and storage conditions using rheological and quantitative chemical analysis techniques such as HPLC (Guaratini et al. 2006; Temova Rakuša et al. 2021). While the negative impacts of ingested caffeine are well documented, the side effects of topical applications require more extensive investigation. Its efficacy and potential toxicity on various skin types as indicated by Baumann Skin Type Indicator (BSTI) and interactions of caffeinated facial creams with other skin care products or medication need to be further investigated (Baumann 2008; Visconti et al. 2020; Gajbhiye and Pal 2024). Ethical issues must be strongly considered following the principles of "four Rs" (Reduction, Refinement, Replacement and Responsibility) for studies that require animal experimentation (Kiani et al. 2022).

Diploknema butyracea (chiuri) plant is primarily found in the sub-Himalayan regions and is native to Nepal, India, and Bhutan. Beside its ethnomedicinal uses, many commercial ventures have explored creating a range of products, including *chyuri* syrup, *chyuri* jam, and *chyuri* cream. Medicinal plants are disappearing rapidly, necessitating effective conservation and sustainable use strategies. Ecological and environmental implications of harvesting chiuri plant need to be considered for sustainable practices and minimize environmental impacts.

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Chitosan nanoparticles encapsulating turmeric (*Curcuma longa*) extract for the management of *Streptococcus agalactiae*-associated breast cancer

CLETUS ANES UKWUBILE¹✉, NNAMDI DAVID MENKITI², OTALU OTALU JR³

¹Department of Pharmacognosy, Faculty of Pharmacy, University of Maiduguri. Maiduguri Rd, 1069 Bama, Maiduguri 600104, Borno, Nigeria.
Tel./fax.: +234-76-231 639, ✉email: doccletus@yahoo.com

²Department of Chemistry, Faculty of Physical Sciences, Ahmadu Bello University. Zaria 810211, Kaduna, Nigeria

³Department of Veterinary Public Health, Faculty of Veterinary Medicine, Ahmadu Bello University. Zaria 810211, Kaduna, Nigeria

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Abstract. Ukwubile CA, Menkiti ND, Otolu Jr O. 2023. Chitosan nanoparticles encapsulating turmeric (*Curcuma longa*) extract for the management of *Streptococcus agalactiae*-associated breast cancer. *Asian J Nat Prod Biochem* 23: 19-26. Breast cancer associated with *Streptococcus agalactiae* Lehmann & Neumann, 1896 infection poses complex therapeutic challenges, often exacerbating inflammatory responses and impacting tumor progression. Despite the use of various anticancer drugs, the development of resistance by cancer cells against these is still prevalent, hence, the need for an appropriate drug delivery strategy in the form of chitosan nanoparticles for effective treatment against breast cancer. This study investigates the antibacterial and anticancer effects of turmeric (*Curcuma longa* L.) extract encapsulated in chitosan nanoparticles (NPs) against *S. agalactiae*-associated breast cancer, assessing its influence on key bacterial and cancer biomarkers. *Curcuma longa* extract-loaded chitosan NPs were synthesized via ionic gelation and characterized for stability and particle size. Breast cancer cells and *S. agalactiae* cultures were treated with these NPs, and bacterial growth inhibition assays quantified antibacterial activity. Anticancer effects were evaluated using cell viability assays and measurements of inflammatory and oxidative stress biomarkers, including Tumor Necrotic Factor- α (TNF- α), interleukin-1 β (IL-1 β), cyclooxygenase-2 (COX-2) and Reactive Oxygen Species (ROS) levels. *Curcuma longa*-loaded chitosan NPs exhibited a significant antibacterial effect, reducing *S. agalactiae* counts by 87% compared to the control ($p < 0.01$). In cancer cells, the NPs decreased TNF- α and IL-1 β levels by 52% and 48%, respectively, and significantly reduced COX-2 expression by 43% ($p < 0.01$). Additionally, ROS levels in treated cancer cells were reduced by 60% compared to control, highlighting the potent antioxidative activity of the NPs. These findings demonstrated the enhanced therapeutic potential of *C. longa*-loaded chitosan NPs for combating infection-associated breast cancer. The encapsulation of *C. longa* extract in chitosan NPs significantly improves antibacterial and anticancer activities, offering a dual-targeted approach that holds promise for treating *S. agalactiae*-associated breast cancer.

Keywords: Biomarkers, breast cancer, chitosan nanoparticles, *Curcuma longa*, *Streptococcus agalactiae*

INTRODUCTION

Breast cancer remains one of the most prevalent malignancies affecting women globally, contributing significantly to morbidity and mortality rates (Tomeh et al. 2019; Ojo et al. 2022). Despite advancements in medical science, breast cancer management presents numerous challenges, particularly when associated with infections by multidrug-resistant organisms like *Streptococcus agalactiae* Lehmann & Neumann, 1896, commonly known as group B *Streptococcus* (GBS) (Mohamad et al. 2018; Akinduti et al. 2022).

Breast cancer remains a leading cause of cancer-related mortality globally, with limited effective therapies for cases complicated by infections such as *S. agalactiae*. Conventional treatment approaches often face challenges such as drug resistance, systemic toxicity, and recurrence. Turmeric (*Curcuma longa* L.) is widely recognized for its anticancer and antimicrobial properties, but its poor bioavailability and rapid metabolism limit its therapeutic efficacy (Khan et al. 2024). Chitosan nanoparticles (NPs) offer a promising solution by improving the stability, bioavailability, and targeted delivery of bioactive

compounds. However, there is a paucity of research on the application of chitosan NPs encapsulating turmeric extract specifically for managing *S. agalactiae*-associated breast cancer. Investigating this targeted approach could address the dual challenge of infection-driven cancer progression and ineffective drug delivery, filling a significant gap in therapeutic development.

More and more, cancer treatments are starting to include natural compounds, and for good reason. These natural substances offer a variety of health benefits and tend to be safer than some synthetic options. This growing interest reflects a desire to find effective treatments that not only target the disease but also consider the well-being of patients. Turmeric, is a popular medicinal plant recognized for its health benefits in both traditional and modern medicine (Hermansyah et al. 2024). One of its key components, curcumin, has powerful properties that reduce inflammation, help fight cancer, and combat infections. The effectiveness of curcumin's anticancer mechanisms is attributed to its ability to disrupt various signaling pathways critical to tumor progression, inhibit cell proliferation, and induce apoptosis, including NF- κ B, STAT3, and PI3K/AKT (Ngulde et al. 2020).

Chitosan, a natural polysaccharide derived from chitin, has the additional advantage of intrinsic antimicrobial activity, which can help combat microbial infections associated with cancer, such as those caused by *S. agalactiae* (Li et al. 2018). The use of chitosan nanoparticles for curcumin encapsulation not only aims to improve curcumin's pharmacokinetics but also provides a dual-action therapeutic approach, targeting both bacterial infection and cancer cells simultaneously. This innovative approach of combining treatments holds great promise for the future of cancer treatment.

The inflammatory environment induced by bacterial infections in breast cancer can accelerate tumor progression and resistance to conventional therapies. *S. agalactiae* has been implicated in eliciting pro-inflammatory responses, leading to the release of cytokines and Reactive Oxygen Species (ROS) that may create an environment conducive to tumor growth and spread (Lukiati et al. 2020). This underscores the need for an integrated treatment approach that can modulate inflammation, directly target microbial infection, and inhibit cancer cell proliferation. Chitosan nanoparticles encapsulating curcumin represent a novel therapeutic modality that can fulfill these requirements by enhancing curcumin's anti-inflammatory and anticancer activities while leveraging chitosan's antimicrobial properties against *S. agalactiae* (Herdiana et al. 2022).

The encapsulation of curcumin in chitosan nanoparticles offers several pharmacological advantages. First, it improves curcumin's stability and prolongs its half-life within the biological system, allowing for sustained therapeutic effects (Ojo et al. 2022). Second, chitosan's cationic nature enables the nanoparticles to interact favorably with negatively charged cell membranes, facilitating the targeted delivery of curcumin to cancer cells (Atwan and Al-Ogaidi 2024; Athar et al. 2024). Moreover, chitosan nanoparticles can penetrate cellular barriers, ensuring that the encapsulated curcumin reaches the intracellular targets involved in cancer cell survival and proliferation (Bahrulolum et al. 2021). Furthermore, the antimicrobial property of chitosan enhances the therapeutic potential by directly combating *S. agalactiae*, thereby potentially reducing the bacterial load and associated inflammatory response (Ugorji et al. 2024).

Nanoparticles can bypass some of these mechanisms by entering cells through endocytosis, thus delivering curcumin directly to intracellular compartments and reducing the likelihood of drug expulsion by efflux pumps (Athar et al. 2024). Chitosan nanoparticles could play a key role in making curcumin more effective against drug-resistant breast cancer cells. This targeted delivery method aims to overcome one of the biggest obstacles in the fight against breast cancer, potentially offering hope for better treatment outcomes (El-Zehery et al. 2022).

This study is driven by the urgent need to find new ways to address two pressing problems: treating breast cancer and treating the bacterial infections that can co-occur with the disease. By exploring innovative strategies, researchers hope to improve treatment and support for those affected by these challenges. Encapsulating curcumin in chitosan nanoparticles is a promising approach that can

deliver a synergistic effect by combining the anticancer, anti-inflammatory, and antimicrobial properties of both curcumin and chitosan (Li et al. 2018; Veselov et al. 2022). This method reflects the latest advancements in nanomedicine, which aim to harness the special characteristics of nanoparticles. By doing this, we can provide more precise and effective treatment while reducing unwanted side effects (Li et al. 2018).

MATERIALS AND METHODS

Preparation of chitosan NPs encapsulating *Curcuma longa* L. extract

Chitosan nanoparticles were synthesized using the ionic gelation method (Deng et al. 2006) as described below:

Preparation of chitosan solution

A 1% (w/v) chitosan solution was prepared by dissolving chitosan in 1% acetic acid under constant stirring for 1 hour at room temperature.

Incorporation of *Curcuma longa* L. extract

A predetermined amount of *C. longa* extract was mixed with the chitosan solution to achieve various concentrations (5%, 10%, and 15% w/v). The mixture was stirred for an additional 30 minutes to ensure homogeneous dispersion.

Formation of nanoparticles

Sodium tripolyphosphate (1% w/v) was added dropwise to the chitosan-extract mixture while stirring continuously for 1 hour to initiate ionic gelation, which led to the formation of nanoparticles. The resulting nanoparticles were centrifuged at 10,000 rpm for 15 min to separate them from the unencapsulated extract, washed three times with distilled water, and lyophilized for storage.

Characterization of chitosan nanoparticles

The synthesized chitosan nanoparticles were characterized (Deng et al. 2006; Jha and Mayanovic 2023) using the following techniques:

Dynamic Light Scattering (DLS)

The size and Polydispersity Index (PDI) of the nanoparticles were determined using a Zetasizer (Malvern Instruments).

Scanning Electron Microscopy (SEM)

The morphology of the nanoparticles was examined using SEM (Jeol JEM-2100). A drop of nanoparticle suspension was placed on a copper grid and dried before imaging.

Zeta potential measurement

The surface charge of the nanoparticles was measured to assess their stability using a Zetasizer.

Encapsulation efficiency

The encapsulation efficiency of curcumin in chitosan nanoparticles was calculated using the formula below:

$$EE (\%) = \frac{\text{Total extract added} - \text{Free extract in supernatant}}{\text{Total extract added}} \times 100$$

Cytotoxicity assay

The cytotoxicity of *C. longa* extract-loaded chitosan nanoparticles against MCF-7 cells was assessed using the MTT assay as described. MCF-7 cells were placed in 96-well plates at a density of 1×10^4 cells per well and incubated overnight at 37°C in an environment with 5% CO₂. Cells were then treated with various concentrations of CSNPs (0, 25, 50, 100, and 200 µg/mL) for 24, 48, and 72 h. After treatment, MTT solution (5 mg/mL) was added to each well and incubated for 4 hours at 37°C. The formazan crystals formed were dissolved in 5 mL of DMSO solution, and the absorbance was measured at 570 nm using a UV-Vis spectrophotometer (Alfuraydi et al. 2024). The cell viability percentage was calculated, and the IC₅₀ value was determined using GraphPad Prism software.

Determination of reactive oxygen species

The Reactive Oxygen Species (ROS) levels were measured using the DCFH-DA assay, which detects intracellular ROS production. After treating MCF-7 cells with CSNPs at concentrations of 0, 25, 50, 100, and 200 µg/mL for 24, 48, and 72 h, cells were incubated with 10 µM DCFH-DA for 30 min at 37°C in the dark. After the incubation period, the cells were rinsed with 5% PBS. Then, we measured the fluorescence intensity, which indicates the levels of Reactive Oxygen Species (ROS), using a microplate reader. This measurement was taken at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The ROS levels were expressed as Relative Fluorescence Units (RFUs) and compared across treatment groups (Zielinska-Blizniewska et al. 2019).

Cancer biomarker analysis

Cancer biomarkers associated with cell proliferation, apoptosis, and oxidative stress, such as BCL-2, BAX, caspase-3, and p53, were analyzed in treated MCF-7 cells. Cells were treated with varying concentrations of CSNPs (0, 25, 50, 100, and 200 µg/mL) for 24, 48, and 72 h. After treatment, cells were lysed, and the supernatant was collected for biomarker analysis (Porter and Jänicke 1999; Cartwright et al. 2017).

ELISA

We utilized ELISA kits to measure the protein levels of BCL-2, BAX, caspase-3, and p53, following the guidelines provided by the manufacturer. The absorbance readings were taken at a wavelength of 450 nm, and we determined the concentrations of each biomarker by referencing standard curves (Ansori et al. 2022; Jaferník et al. 2023).

Western blot

In a separate set of experiments, protein expression of cancer biomarkers was further validated by western blotting. We started by separating the cell lysates using SDS-PAGE, after which we transferred the proteins to PVDF membranes. Next, we used primary antibodies specifically targeting BCL-2 to probe these membranes, as

well as BAX, caspase-3, and p53, followed by HRP-conjugated secondary antibodies. Bands were visualized using Enhanced Chemiluminescence (ECL), and protein levels were quantified by densitometry analysis (Amalraj et al. 2017).

Evaluation of antibacterial activity

The antibacterial efficacy of the nanoparticles was assessed through the disc diffusion method and broth microdilution techniques. In this procedure, bacterial suspension of *S. agalactiae* was prepared and adjusted to a concentration of 1×10^8 CFU/mL. Blood agar plates were inoculated with the suspension, and sterile filter paper discs were impregnated with different concentrations of CSNPs (0, 10, 25, 50, 100 µg/mL) and placed on the agar surface. The plates were incubated at 37°C for 24 h, and the diameter zones of inhibition were measured using a transparent rule. Serial dilutions of CSNPs were prepared in broth, ranging from 1000 µg/mL to 15.625 µg/mL. The bacterial suspension was added to each dilution and incubated at 37°C for 24 hours. The lowest concentration of CSNPs that inhibited visible bacterial growth was recorded as the MIC (Bisen 2014).

Statistical analysis

The data are presented as mean ± SD (n = 3) from three separate experiments. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's post-hoc test. A p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Characterization of chitosan NPs

The result below summarizes the physicochemical properties and release characteristics of *C. longa* extract-loaded Chitosan Nanoparticles (CSNPs). The nanoparticles have a uniform particle size of 145 nm and a low PDI (0.21), indicating a narrow distribution suitable for controlled release. The positive zeta potential (+28.3 mV) suggests good colloidal stability, reducing aggregation in suspension. High encapsulation efficiency (82.5%) and yield (85.75%) reflect efficient loading and synthesis. The swelling index (1.85) shows the degree of water absorption influencing release rates. Experiments conducted at a physiological temperature (37°C) indicate a sustained release profile, with a 24-hour cumulative release of 78.65% and diffusion-controlled release following the Higuchi model, supporting the potential for prolonged therapeutic action (Table 1).

Cytotoxicity of CNPs on MCF-7 cells

The result is presents a concentration-dependent decrease in MCF-7 cell viability over 24, 48, and 72 h after treatment with *C. longa* extract-loaded CSNPs (Table 2). The progressive decrease in cell viability as the concentration of nanoparticles increases is a significant finding. As the concentration of nanoparticles increases, cell viability progressively decreases. Notably, at a

concentration of 100 $\mu\text{g/mL}$, cell viability is reduced to 35.00% after 72 hours, while curcumin reduced it to 25.30%, suggesting that curcumin is slightly more effective at inhibiting cell growth at higher concentrations, indicating strong cytotoxicity. The calculated IC_{50} value was calculated to be 62.00 $\mu\text{g/mL}$, further supporting this, demonstrating that this concentration effectively inhibits 50% of cell viability. This data supports the therapeutic potential of *C. longa* extract-loaded CSNPs in targeting breast cancer cells by inhibiting their growth in a time and dose-dependent manner to contribute to the development of new treatments for breast cancer, fostering hope and encouragement for the future (Table 1).

Effects on cancer biomarkers

The study on the relative expression levels of Bcl-2, Bax, caspase-3, and p53 proteins in response to chitosan NPs (CSNP) concentration and incubation time, the results have significant implications for apoptosis. For instance, the decrease in Bcl-2 levels with higher CSNP concentration suggests a potential reduction in anti-apoptotic signaling, while the increase in Bax levels implies an enhancement in pro-apoptotic activity. The notable increase in caspase-3 levels at higher concentrations and longer incubation times suggests a potential for apoptosis induction, and the significant increase in p53 levels at higher concentrations and longer times indicates the activation of tumor-suppressor pathways (Figure 1).

Table 1. Characterization of *Curcuma longa* L. loaded CSNPs

Parameter	Value/unit
Particle size	145.00 $\pm$ 5.00 nm
Polydispersity index (PDI)	0.21
Zeta potential	+28.30 $\pm$ 1.80 mV
Encapsulation efficiency	82.50 $\pm$ 3.90%
% yield	85.75 $\pm$ 2.50%
Swelling index	1.85 $\pm$ 0.12
Temperature	37.00°C
Drug kinetics model	Higuchi
Cumulative drug release (24 h)	78.65 $\pm$ 2.35%

Note: Results are mean $\pm$ SD (n = 3)

Table 2. Cytotoxicity of *Curcuma longa* L. extract-loaded CSNPs on MCF-7 cells

Concentration ($\mu\text{g/mL}$)	% cell viability (24h)	% cell viability (48h)	% cell viability (72h)
0 (control)	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00
25	85.20 $\pm$ 3.50	75.30 $\pm$ 4.00	60.50 $\pm$ 4.80
50	70.10 $\pm$ 4.20	55.40 $\pm$ 3.90	45.20 $\pm$ 4.10
100	55.30 $\pm$ 4.60	42.50 $\pm$ 4.00	35.00 $\pm$ 4.30
200	40.50 $\pm$ 4.80	30.20 $\pm$ 3.70	20.10 $\pm$ 3.50
*Curcumin(100)	45.50 $\pm$ 3.90	35.20 $\pm$ 4.10	25.30 $\pm$ 3.80

Note: Results are mean $\pm$ SD (n = 3). * = Standard drug used as a positive control

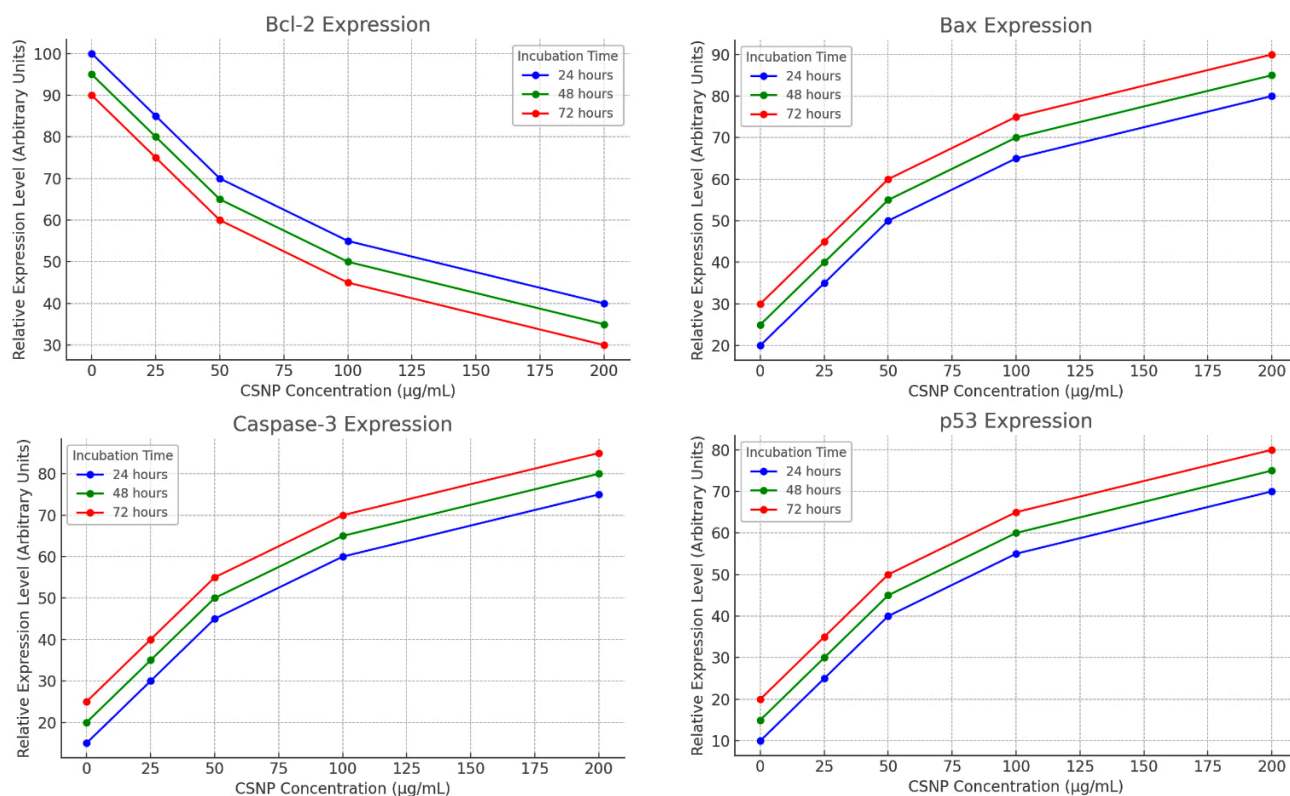


Figure 1. Relative expression levels of Bcl-2, Bax, caspase-3, and p53 proteins as a function of Chitosan NPs (CSNP) concentration and incubation time. Values represent normalized protein expression levels as a percentage relative to control

Similarly, the results in terms of intensities the standard drug row, show the band intensities for each protein after treatment with the standard drug. The values for Bcl-2, Bax, caspase-3, and p53 are between the control and higher treatment concentrations, indicating a moderate effect. These findings have significant implications for our understanding of drug treatments. For example, the Bcl-2 intensity under the standard drug condition is 0.85, showing a slight reduction compared to the control, while Bax, caspase-3, and p53 intensities are higher than the control, indicating some activation of apoptosis and tumor-suppressive pathways, like the trend observed in the treatment groups (Table 3).

Western blot image representation

The Western blot results show protein expression patterns in cells treated with varying concentrations of CSNPs (Chitosan NPs) compared to untreated control cells. Lane 1 represents the control (untreated cells), where the protein bands for Bcl-2, Bax, caspase-3, and p53 are at baseline levels (intensity = 1.0). Lane 2 (CSNPs 25 $\mu\text{g/mL}$) shows slight increases in Bax, Caspase-3, and p53 expression, with a moderate reduction in Bcl-2 expression, suggesting early activation of apoptosis pathways. Lane 3 (CSNPs 50 $\mu\text{g/mL}$) demonstrates further increases in Bax, caspase-3, and p53 expression, along with a continued decrease in Bcl-2 expression, indicating a stronger apoptotic response. Lane 4 (CSNPs 100 $\mu\text{g/mL}$) shows even higher expression levels of Bax, Caspase-3, and p53, with Bcl-2 further reduced, suggesting a pronounced activation of apoptosis. Lane 5 (CSNPs 200 $\mu\text{g/mL}$) exhibits the highest expression of Bax, caspase-3, and p53, with a significant decrease in Bcl-2, supporting the notion that higher concentrations of CSNPs induce a potent apoptotic response through the upregulation of pro-apoptotic proteins and the downregulation of anti-apoptotic proteins. This pattern aligns with the densitometry data, indicating that CSNPs promote apoptosis in a concentration-dependent manner (Figure 2).

Antibacterial activity against *S. agalactiae*

The antibacterial activity of Chitosan Nanoparticles (CSNPs) against *S. agalactiae* was assessed using the disc diffusion method. The results showed that the zones of inhibition increased with the concentration of CSNPs, with the largest inhibition zone (20.00 ± 2.0 mm) observed at

100 $\mu\text{g/mL}$. The extract without chitosan, at a concentration of 50 $\mu\text{g/mL}$, showed a moderate inhibition of 12.00 ± 1.2 mm, while the CSNPs demonstrated stronger antibacterial effects at higher concentrations, with 15.00 ± 1.0 mm at 50 $\mu\text{g/mL}$, 18.00 ± 1.8 mm at 75 $\mu\text{g/mL}$, and 20.00 ± 2.0 mm at 100 $\mu\text{g/mL}$. The standard antibiotic (Penicillin) showed the largest zone of inhibition at 30.00 ± 1.5 mm. The Minimum Inhibitory Concentration (MIC) for CSNPs was determined to be 50 $\mu\text{g/mL}$ (Ojo et al. 2022). These results suggest that while CSNPs show significant antibacterial activity, the inclusion of chitosan enhances the antibacterial effect compared to the extract alone (Table 4).

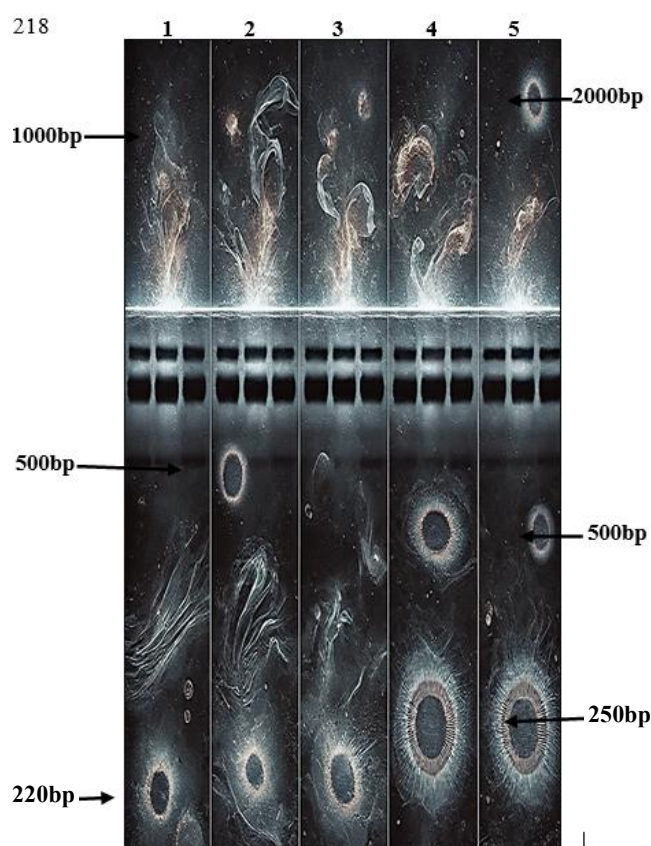


Figure 2. Western blot analysis of protein expression in response to CSNPs treatment: Bcl-2, Bax, caspase-3, and tumor protein 53 (p53) at varying concentrations

Table 3. Densitometry analysis of protein expression in response to treatment concentrations Bcl-2, BAX, caspase-3, and p53 intensities

Treatment ($\mu\text{g/mL}$)	Bcl-2 intensity	Bax intensity	Caspase-3 intensity	p53 intensity
0 (control)	1.0	1.0	1.0	1.0
Standard drug	0.85	1.25	1.5	1.2
25	0.9	1.2	1.3	1.1
50	0.75	1.4	1.6	1.25
100	0.5	1.6	1.8	1.4
200	0.3	1.8	2.0	1.6

Note: Values represent relative band intensities, normalized to control (0 $\mu\text{g/mL}$)

Table 4. Antibacterial activity of Chitosan NPs (CSNPs) against *S. agalactiae*

Sample/concentration	Zone of inhibition (mm)
Extract without chitosan (50 $\mu\text{g/mL}$)	12.00 ± 1.2
Chitosan NPs (25 $\mu\text{g/mL}$)	10.00 ± 1.5
Chitosan NPs (50 $\mu\text{g/mL}$)	15.00 ± 1.0
Chitosan NPs (75 $\mu\text{g/mL}$)	18.00 ± 1.8
Chitosan NPs (100 $\mu\text{g/mL}$)	20.00 ± 2.0
Standard antibiotic (Penicillin)	30.00 ± 1.5

Note: Results are mean $\pm$ SD (n = 3)

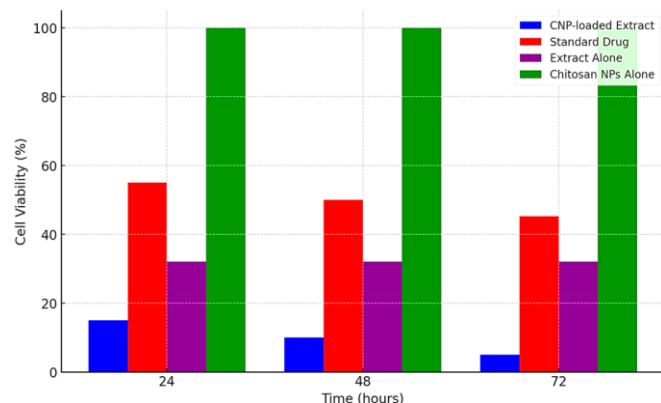


Figure 3. Cytotoxicity of chitosan NPs on MCF-7 breast cancer cells at 24, 48, and 72 hours. The CNP-loaded extract demonstrates significantly ($p < 0.01$) lower cell viability, particularly at the 72-hour mark

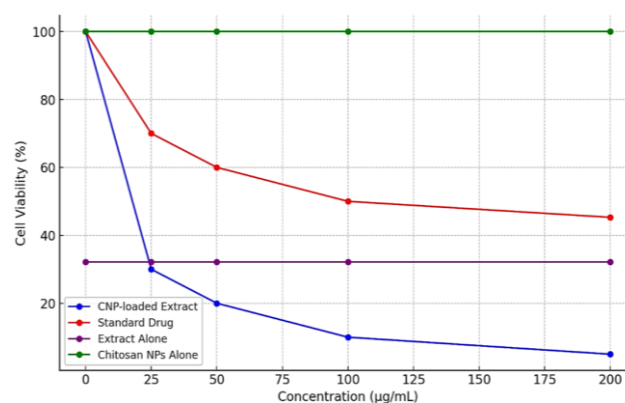


Figure 4. Effect of the treatments on MCF-7 cell viability, comparing different concentrations (0, 25, 50, 100, and 200 µg/mL). Results are mean $\pm$ SD ($n = 3$)

Comparative cytotoxicity effects

The results demonstrates the cytotoxic effects of different treatments on MCF-7 cells over time at 24, 48, and 72 hours. The treatments include Chitosan Nanoparticles (CSNPs) loaded with extract, the extract alone, the standard anticancer drug, and chitosan NPs alone yield promising results. The CSNP-loaded extract, with its highest cytotoxicity reflected by the lowest cell viability at each time point, particularly reaching only 5% viability by 72 h holds great promise. The standard drug, with its moderate cytotoxicity, maintaining around 45% viability at the final time point, also shows potential. The extract alone and chitosan nanoparticles without the extract demonstrate significantly less cytotoxicity, with extract alone at 32.12% viability and chitosan nanoparticles showing minimal effects on cell viability, around 100% throughout (Figure 3).

Cancer cell viability

The results show the concentration-dependent effect of the treatments on MCF-7 cell viability, comparing different concentrations (0, 25, 50, 100, and 200 µg/mL). It's important to note that the CSNP-loaded extract shows a clear concentration-dependent decrease in cell viability, achieving the greatest reduction in viability at the highest concentration (200 µg/mL) with only 5% cell viability. The standard drug also displays concentration-dependent effects but with less impact, maintaining around 45.3% viability at 200 µg/mL. The extract alone and chitosan NPs show minimal cytotoxicity at all concentrations, indicating that the chitosan carrier itself does not contribute significantly to cytotoxicity without the active extract. These findings suggest that CSNP-loaded extract is highly effective in reducing MCF-7 cell viability, potentially offering an enhanced cytotoxic effect over the standard drug. This information about the concentration-dependent effects of the treatments can guide future research and potential applications (Figure 4).

Discussion

This study highlights the potential of Chitosan Nanoparticles (CSNPs) encapsulating *C. longa* extract as a promising and innovative therapeutic approach for managing *S. agalactiae*-associated breast cancer. The successful synthesis and detailed characterization of CSNPs (Table 1) confirm their suitability for drug delivery applications, especially in enhancing the bioavailability and efficacy of curcumin. CSNPs offer a biodegradable and biocompatible vehicle for the encapsulation of bioactive compounds, overcoming many of the inherent limitations of curcumin, such as poor solubility, low bioavailability, and rapid metabolic degradation (Li et al. 2018; Herdiana et al. 2022). The encapsulation of curcumin within CSNPs ensures its controlled release and localized delivery, reducing systemic side effects while maintaining therapeutic efficacy (Li et al. 2018).

The cytotoxicity results (Table 2) demonstrate that CSNPs significantly reduce the viability of MCF-7 cells in a concentration-dependent manner, with an IC_{50} value of 62 µg/mL. This result is consistent with previous studies that have documented the antiproliferative and cytotoxic effects of curcumin on breast cancer cells (Ojo et al. 2022). The increased effectiveness of the CSNP formulation in killing cancer cells can be largely attributed to its ability to improve the solubility of curcumin and enhance its uptake by cells. This is a common advantage of using nanoparticles for drug delivery. By allowing curcumin to reach higher concentrations inside the cells, the formulation boosts its anticancer effects. Moreover, the nanoparticles are particularly good at getting through the cell membrane, making the treatment even more effective potentially leading to better therapeutic outcomes when compared to free curcumin (Ojo et al. 2022). The concentration-dependent reduction in cell viability supports the idea that CSNPs deliver curcumin in a way that maximizes its therapeutic impact while minimizing toxicity to healthy cells (Figures 1 and 2; Table 3).

The impressive ability of CSNPs to lower the viability (Figures 3 and 4) of MCF-7 cancer cells suggests they could be a strong alternative to traditional chemotherapy. Curcumin, a promising cancer-fighting agent, has already shown promise in fighting cancer. When combined with CSNPs, its effectiveness is significantly enhanced, providing a more targeted treatment. Unlike conventional chemotherapy, which often affects healthy cells and leads to side effects, CSNPs excel in delivering curcumin directly to the tumor cells. This targeted delivery not only minimizes potential toxicity but also improves the overall effectiveness of the treatment (Herdiana et al. 2022). This targeted delivery may help lessen the negative side effects commonly linked to chemotherapy, including nausea, fatigue, and hair loss—significant worries for breast cancer patients receiving treatment.

Furthermore, the antibacterial activity of CSNPs against *S. agalactiae* (Table 4) is noteworthy, particularly because this bacterium has been implicated in the pathogenesis of breast cancer. Chronic infections caused by pathogens like *S. agalactiae* contribute to inflammatory pathways that promote cancer cell proliferation and metastasis (Ojo et al. 2022). The effective inhibition of *S. agalactiae*, demonstrated by the zones of inhibition and the Minimum Inhibitory Concentration (MIC) value, underscores the dual therapeutic potential of this CSNP formulation. The antibacterial effects of curcumin have been well-documented, with numerous studies highlighting its ability to inhibit the growth of various pathogenic bacteria, including *S. agalactiae* (Mongalo et al. 2019; Khalid et al. 2022; Oyinlola et al. 2024). This research builds on previous findings by demonstrating that curcumin when contained in CSNPs, boosts its antibacterial effectiveness. This could have important consequences for treating breast cancer patients, particularly those facing infections that hinder their cancer progression.

The dual-action effect of CSNPs - targeting both cancerous cells and bacterial infections—offers a novel approach to managing breast cancer (Figure 3). This approach goes beyond just fighting the tumor; it also tackles infections that might contribute to cancer coming back. By combining the cancer-fighting and infection-fighting strengths of curcumin, we have a chance to improve treatment results by addressing both the cancer itself and any underlying infections at the same time (van Haaften et al. 2011; Ojo et al. 2022). Hence, using CSNPs as a delivery method, we can help curcumin more effectively target cancer cells and harmful bacteria. This targeted approach not only makes the treatment more precise but also promises to lower the chances of cancer coming back and improve long-term survival outcomes in breast cancer patients.

Further investigation into the in vivo safety and efficacy of CSNPs loaded with *C. longa* extract in animal models is critical. While the results from in vitro studies are promising, animal studies are essential to confirm the pharmacokinetics, biodistribution, and toxicity profiles of CSNPs before clinical translation. Understanding the mechanisms that underpin the observed antibacterial and anticancer activities of CSNPs will also be key to

optimizing this formulation for clinical use. Previous studies have shown that nanoparticles, including CSNPs, can interact with both cancer cells and bacterial pathogens at the molecular level, influencing cellular signaling pathways involved in proliferation, apoptosis, and immune responses (van Haaften et al. 2011; Ralte et al. 2022; Malviya et al. 2023). In summary, this study highlights the exciting potential of using curcumin-loaded CSNPs as a treatment for breast cancer linked to *S. agalactiae*. The innovative delivery method not only boosts the anticancer effects but also combats bacterial infections, offering hope for better outcomes for patients dealing with both issues. To truly realize the benefits of this approach, more research, including animal studies and clinical trials, is necessary. With further exploration, CSNPs could become a valuable tool in cancer therapy, paving the way for improved care in everyday medical practice.

In conclusion, this study highlights the potential of chitosan nanoparticles encapsulating *C. longa* extract as a promising therapeutic approach for managing *S. agalactiae*-associated breast cancer. The nanoparticle formulation significantly enhances the bioavailability and targeted delivery of turmeric's bioactive compounds, leading to improved anticancer and antimicrobial efficacy. The findings underscore the dual therapeutic potential of the formulation in combating infection-driven cancer progression while minimizing systemic toxicity. Prospects include further in vivo studies to validate the safety and efficacy of this approach, as well as exploring its application against other multidrug-resistant pathogens and infection-related cancers. This research sets a foundation for the development of advanced nanoparticle-based combination therapies, paving the way for personalized medicine and targeted drug delivery systems in oncology.

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Harnessing L-asparaginase from Solanaceae plants: Purification and molecular dynamics studies for cancer targeting

AMBREEN AISHA^{1*}, AYESHA SAJJAD², NAYLA MUNAWAR³, HGAFIZ MUHAMMAD HUSNAIN AZAM⁴,
UZAIR ISHTIAQ⁵, SHAISTA NAWAZ⁶

¹Department of Biochemistry, Faisalabad Medical University. 38000 Faisalabad, Pakistan. Tel.: +92-41-9210080, *email: aishafmu@gmail.com

²Department of Microbiology, Amna Inayat Medical College. Faizpur Interchange, M2 Motorway, Lahore, Pakistan

³Department of Chemistry, United Arab Emirates University. Al-Ain 15551, United Arab Emirates

⁴Institute of Biotechnology, Faculty Environment and Natural Sciences, Brandenburg University of Technology Cottbus-Senftenberg. Universitätsplatz 1, 01968 Senftenberg, Germany

⁵Department of Research and Development, Paktex Industries. 2.5 KM Tatlay Road, Saroya Abad, Kamoke, Gujranwala, 52470, Pakistan

⁶Food and Biotechnology Research Center, Pakistan Council of Scientific and Industrial Research (PCSIR). Lahore 54600, Pakistan

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Abstract. Aisha A, Sajjad A, Munawar N, Azam HMH, Ishtiaq U, Nawaz S. 2025. Harnessing L-asparaginase from Solanaceae plants: Purification and molecular dynamics studies for cancer targeting. *Asian J Nat Prod Biochem* 23: 27-37. Conventional chemotherapeutic regimens have their adverse effects. Our research, however, offers a potential breakthrough in cancer treatment. We have discovered that L-asparaginase from plant sources can selectively target L-asparagine, the nutritional requirement of cancer cells. The aim was to elucidate the function and mechanism of action of anticancer and bactericidal plant-sourced L-asparaginase protein via proteomics. Biochemical, chromatographic techniques and SDS PAGE were employed for purifying L-asparaginase from Solanaceae plants, including *Datura innoxia*, *Atropa belladonna*, *Hyoscyamus niger* (leaves), *Lycopersicum esculentum* (fruit), *Solanum tuberosum* (roots), and *Ipomoea batatas* (roots). Michaelis-Menten and Lineweaver Burk's plot demonstrated the characterization of the extracted protein from *D. innoxia*, which exhibited the maximum enzyme activity. Further, molecular dynamics simulations were employed to establish antimicrobial efficiency (through potato disc assays) and to determine the antitumor efficiency. Kinetic studies of L-asparaginase from *D. innoxia* revealed optimal activity at pH 8-8.5 and 37°C with [S]=8 ug/mL, Km 0.1766 umol provided 1/2V max 0.525 umol/min, while the protein band of approximately 30 kDa. Ethanolic extracts inhibited growth 54.5, 33.30, and 45.65% among *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*, respectively, with MIC 6.25 mg/dL. L-asparaginase anticancer mediation was validated using molecular dynamics simulations (InterPro Scan), identifying novel domain IPR027474 and active sites IPR020827 and IPR027475. This mechanism provided insights into anticancer action and potential sources for combinational therapies in clinical oncology.

Keywords: Characterization L-asparaginase, chemotherapeutic, chromatography, conventional pharmaceuticals, extraction, purification

INTRODUCTION

Cancer and bacterial infections are two major health concerns worldwide, affecting millions every year. Natural products derived from plants, fungi, and microorganisms have been widely studied for their anticancer properties. These compounds exhibit a range of mechanisms of action, including induction of apoptosis, inhibition of cell proliferation, and suppression of angiogenesis (Newman and Cragg 2016). Several alternatives to conventional drugs, such as taxol, vincristine, and camptothecin, have been used as chemotherapy drugs to treat cancer (Cragg and Newman 2013). In recent years, there has been increasing interest in the potential of natural products as sources of new anticancer drugs. For example, curcumin, a compound found in turmeric, has been shown to have anticancer properties and is being studied as a potential anticancer drug (Aggarwal et al. 2009).

Bacterial infections are a major health concern with the emergence of antibiotic-resistant bacteria posing a significant challenge to the development of new antibacterial drugs. Natural products derived from plants,

fungi, and microorganisms have been studied for their antibacterial properties, with several compounds showing promising results in preclinical studies (Newman and Cragg 2016). For example, berberine, a compound found in several plants, has been shown to have antibacterial properties against a range of bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) (Stermitz et al. 2000).

Switching from antibiotics and chemotherapy to non-synthetic herbal medicines has revolutionized the world. Medicinal plants provide the basic raw materials for indigenous pharmaceuticals (Chamani et al. 2020). Medicinal plants used to treat chronic diseases have attracted the interest of scientists. The extracts of various medicinal plants have been used as medicine (Turrini et al. 2014). There are nearly 2300 plant species in the Solanaceae family, including medicinal plants like *Solanum nigrum*, *Withania somnifera*, *Capsicum annuum*, and tomato with numerous biological activities, i.e., antifungal, antibacterial, antioxidant, cytotoxic, and antitumor activities (Rani et al. 2021).

Solanaceae family comprises excellent therapeutic plants such as *S. nigrum* (black nightshade), potato (*Solanum tuberosum*), tomato, and other medicinal plants. The members of Solanaceae have already been exploited and confirmed their antibacterial and antitumoral potential, i.e., *W. somnifera*, *C. annuum*, *S. nigrum*, *S. tuberosum* and *Solanum lycopersicum* (Ambreen et al. 2019). Various research reports have widely investigated the potential of potato peels to produce useful compounds, particularly phenolic compounds, which are highly demanded in the industrial sector (Mushtaq et al. 2023, 2024a,b). Likewise, plants containing therapeutically bioactive agents, including *Atropa belladonna* L. (deadly nightshade), *Datura stramonium* L. (Jimsonweed), and *Hyoscyamus niger* L. (nightshades), require moist soil habitat to grow. These weeds can also grow in shallow and stony soil. They are cultivated in tropical and subtropical regions of agro climate and widely consumed as medicine for their antioxidant, anti-inflammatory, hepato-protective, diuretic, antitumorogenic, and antipyretic properties. They comprise a diversified spectrum of potentially active metabolites against ailments (Ayo 2010; Yadav et al. 2022). *Ipomoea batatas* are known scientifically for their antioxidant, anti-diabetic, wound healing, anti-ulcer, antibacterial, and anti-mutagenic activities. It is also an immune booster for relieving gastrointestinal and upper respiratory symptoms (Panda et al. 2011; Panda et al. 2012). *Lycopersicum esculentum* also has a high amount of lycopene and fewer glycoalkaloids. To date, various studies have demonstrated several benefits of these bioactive and antioxidant compounds, namely anticarcinogenic, anti-inflammatory, cardioprotective, hepatoprotective, and antibacterial properties (Pinela et al. 2016). *Atropa belladonna* is pre-declared as a primary commercial source of alkaloid drugs, i.e., atropine. Its antibacterial and antimicrobial properties also scavenge cancer cells by imposing a high impact of antioxidants (EL-shaer and Ibrahim 2021). *Hyoscyamus niger* is well known for its antimicrobial, antispasmodic, hypotensive, convulsant, anti-inflammatory, antipyretic, and analgesic characteristics (Begum 2010).

The current study investigates a novel source of L-asparaginase from the Solanaceae family to minimize immunological reactions induced by the microbial-sourced enzyme (Aisha et al. 2022). L-asparaginase (EC3.5.1.1) is an enzyme of medical prominence and is used as a chemotherapeutic agent against acute lymphoblastic leukemia and other cancers. It also possesses immense potential to cure autoimmune and infectious diseases. The vast applications of this enzyme in the healthcare sector have increased its market demand (Vimal and Kumar 2017). The L-asparaginase (EC 3.5.1.1) is used in chemotherapeutic combinations in cancer management to treat leukemia. Crude extracts of medicinal plants of the Solanaceae family, like *W. somnifera*, *S. nigrum*, and *C. annuum*, have been investigated for anticancer activity and then formulated for oncology (Ogunsuyi et al. 2022). The present study's objective is to extract and purify a commercially high titer of the enzyme L-asparaginase through stratified chromatographic methodology from inexpensive plant sources to meet its therapeutic needs in

cancer treatment and biotechnology research.

MATERIALS AND METHODS

Chemicals

The chemicals used in this study were L-Asparagine (Sigma Aldrich), Sephadex G-100 (Sigma Aldrich), DEAE cellulose resin (Diethylamineethylamine), and dialyzing membrane with a pore size of 3.2 microns was purchased from Shenzhen Taoshi Co., Ltd., Japan. SDS (Sodium dodecyl sulfate), 30% acrylamide stock (37.5: 1 acrylamide: bisacrylamide) (Bio-Rad Laboratories), TEMED (Life Technologies, Gibco®), ammonium persulfate (Sigma Aldrich), pre-stain protein M.W. marker (Bio-Rad Laboratories), bromophenol blue (Thermo Fisher Scientific), tris base (Calbiochem-Behring), Tris-HCl (pH 6.8), and β -mercaptoethanol (Sigma Aldrich).

Microbial strains

Escherichia coli strain ST131 KX171170–171195 (Ali et al. 2019), *Staphylococcus aureus* strain 32S ST 239 JTJX000000000 (Khan et al. 2016) and *Bacillus subtilis* strain DH5 α accession number AJ004803 (Soldo et al. 2002).

Plant samples for enzyme extraction

Tubers of *S. tuberosum*, *I. batatas*, and fruit of *L. esculentum* were purchased from the local market of Faisalabad, Pakistan. In contrast, leaves and fruit of *D. innoxia*, *A. belladonna* (deadly nightshade), and *H. niger* (nightshades) were obtained from a botanical garden situated at the University of Agriculture Faisalabad, Punjab, Pakistan.

Crude extract preparation

The leaf extracts of *A. belladonna* (deadly nightshade), *H. niger* (nightshades), *L. esculentum*, and *D. innoxia*, as well as root extracts of potato and sweet potato, were prepared after milling in a solution of 0.2 M phosphate buffer at pH 8.6 (Jamil et al. 2007). The crude protein extract was precipitated by different saturation percentages, initially 50-60%, 70-80%, and finally 90-100% of ammonium sulfate (Jan et al. 2022). The sample was kept at -20°C overnight to precipitate the protein. The 80% saturated ammonium sulfate solution produced a pellet after centrifugation at 8217-28341 RCF for 30 min at 4°C. The pellet was subjected to protein analysis and dialyzed against distilled water for 24h at 4°C while discarding the supernatant. The protein sample was kept at -20°C for further bioassay and purification (Kirar et al. 2022).

Enzyme purification

The Biuret method determined the protein content of crude enzymes partially purified, dialyzed, and pure forms at 540 nm (Gornall et al. 1949). The L-asparaginase enzyme was partially purified by dialysis. The supernatant-containing enzyme was loaded on DEAE cellulose column anion exchange chromatography using phosphate buffer pH 6.8 (Kaur and Arora 2009; Rahman et al. 2009). Almost 50

fractions were collected at the flow rate of 30 mL/hour (Jamil et al. 2007). Those fractions with high protein content and enzyme activity were loaded on a gel filtration column to obtain purified enzymes for therapeutic application. Analytical grade Sephadex G-100 was used as porous material buffered with 0.1 M Tris- HCl buffer to equilibrate the column at pH 8.6 (Dixit et al. 2013). The enzymatic analysis was performed by nesslerization at wavelength 450 nm (Moharib 2018). Each extraction and purification part was replicated in triplicate analysis.

Enzyme and protein purification

The protein content of enzymes in crude form, partially purified shape, dialyzed form, and purified form was determined by (Gornall et al. 1949) using B.S.A. as standard. Absorbance (O.D) of protein was obtained at the 540 nm wavelength. The enzyme was assayed according to the nesslerization method by converting L-asparagine to ammonia and L-aspartate at 37°C and pH 8.5 under a specific condition (Ren et al. 2010).

Kinetic characterization of enzymes

The highest protein and enzyme content was found in six selected plants of the family Solanaceae, and L-asparaginase-bearing plant tissue was chosen for further characterization (Moharib 2018). The effect of pH (4-9) and substrate was recorded on the activity of purified L-asparaginase obtained from the plant extracts that exhibited the highest titer of the enzyme. The reaction mixture of the enzyme was also assayed at different temperatures at 15-65°C, and the substrate (asparagine) concentration was 2-14 µg/mL (Moharib 2018). Different buffer solutions were prepared to estimate the optimal pH for L-asparaginase. Potassium phosphate, sodium acetate, and Tris -HCl buffers were used to design a pH range from 3-9. For kinetic studies, the substrate 0.01 mMol L-asparagine was added to 0.2 M buffer solutions at a specified pH. The purified enzyme was incubated at different pH values 3-9 at 37°C. Km and Vmax were resolved through enzyme kinetic tools such as the Lineweaver-Burk plot and the Michaelis-Menten plot using the following equations:

$$V_o = V_{max}[S] / K_m + [S] \text{ -- Michaelis Menton eq.}$$

$$1/V_o = K_m / V_{max}[S] + 1/V_{max} \text{ -- Line Weaver Burk eq.}$$

Where:

V_o: Initial velocity

V_{max}: Maximum velocity

[S]: Substrate concentration,

K_m: Substrate concentration at ½ V_{max}

Molecular weight by SDS PAGE

Electrophoresis was done on the purified enzyme from Gel filtration chromatography by following Laemmli et al. (1970) discontinuous SDS-PAGE system with a 15% acrylamide gel. The electrophoresis was performed at 100V. The gels were stained for 45 min in a staining solution of 6% acetic acid, 500 mL of H₂O, 44% methanol, and 2.25 g of Coomassie brilliant blue. Destaining was performed in 20% methanol, 5% acetic acid, and 750 mL

of distilled water. This process continued until the background color vanished, and protein bands could easily be seen.

Antibacterial assay

The antibacterial action of crude extract of *D. innoxia* leaves was assessed through standard disc diffusion method (A.S.T.) antibiotic susceptibility testing. The stock solution was prepared by mixing 25 mg of the crude extracts of water and ethanol dissolved in 1 mL of DMSO and was applied on the respective discs. *Bacillus subtilis*, *S. aureus* gram (+), and *E. coli* gram (-) were treated against extracts of *D. innoxia* leaves extract. Observations were recorded and compared with the standard antibiotic of Erythromycin 250 mg. After 24 h of incubation, the noticeable diameter of clear zones of inhibition was measured, which confirmed antimicrobial effects, and percentage inhibition was calculated (Mannan et al. 2014; Noor et al. 2014; Ali et al. 2019; Qasim et al. 2020).

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of crude extracts was determined by the agar dilution method. First, 18 mL of nutritional agar was autoclaved, 2 mL of the crude extract at concentration of 50-0.78 mg/mL was added to each test tube, which was then poured into pre-labeled petri dishes. An extra petri dish with only nutrient agar was prepared as a control. The bacterial strain was adjusted to 0.5 McFarland turbidity standard and transferred to each plate with different concentrations of crude extract. The plates were incubated at 37°C for 24h and the MIC was determined as the minor concentration that inhibited the growth of the respective organism. Next, to determine the minimum bactericidal concentration (MBC), the broth used in MIC was streaked on agar plates, and the MBC was defined as the first dilution where no growth was observed. For the serial dilution, 1 mL of the stock solution of 50 mg extract/mL of DMSO was added to 8 vials with 1 mL of DMSO in each vial. The extract was then serially diluted into 7 vials to obtain 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mg/mL conc. The study found that the crude extract had bactericidal activity against the tested bacterial strains (Janani et al. 2019).

Statistical analysis

The data were subjected to statistical analysis, using Student's t-test to compare variables. The data are expressed as means ± S.D.

RESULTS AND DISCUSSION

The homogenates of six selected plants revealed L-asparaginase activity. *Datura innoxia* and *I. batatas* exhibited pronounced enzyme activity 56.311, 46.93U/mL in crude extracts along with statistically highly significant values by T-test as shown in Figure 1. The enzyme activity of 44.18 and 24.93 U/mL were obtained from the crude extracts of *L. esculentum* and *A. belladonna*, respectively.

In contrast, *H. niger* and *S. tuberosum* extracts revealed enzyme activity as 10.38 U/mL and 10.98 U/mL, respectively.

The purification summaries of all plants are expressed in Figure 1. The partially purified form of the enzyme sample of 500 uL was used for Gel filtration by Sephadex G-100, which resulted in purified enzyme for therapeutic grade for six selected plants (fruit). After gel filtration, the enzyme purified from *D. innoxia* yielded 56.77%, and the mean activity of the crude extract was 30.14±0.928 U/mL (Table 1). Likewise, the specific activity of L-asparaginase was significantly higher ($p<0.05$) in *D. innoxia* (135.25 U/mg) and *A. belladonna* (170.39 U/mg) as compared to other plant extract's specific activity given in Figure 2. At the same time, the percentage yield was 56.77, 53.4, 56.42, 44.3, and 52.3%, respectively.

Figures 3, 4, 5, 6, and 7 depict the summarized view of six plants' activities and specific activities (left side of the graph) with the amount of protein extracted (right side of the graph) at each purification step, i.e., ammonium sulfate precipitation, dialysis, ion exchange, and gel filtration. Among all selected plants, *D. innoxia* proved the maximum activity of L-Asparaginase; its ion exchange and gel filtration curves are explained in Figures 8.B and 8.C. In contrast, enzyme activity at each purification step is expressed by a two-way graph, as shown in Figure 8.A.

Molecular weight by SDS PAGE

The highest enzyme titer-sharing sample from *D. innoxia* was introduced to the SDS PAGE. It showed promising results of L-asparaginase with a maximum protein of 30±1.5 kDa comparable with the standard marker proteins of known molecular weight (Figure 8.D).

L-asparaginase activity affected by kinetic parameters

To check enzyme stability purified L-asparaginase was incubated at different physiological pH to obtain the optimal milieu for enzyme efficacy. At pH 8-8.5, the enzyme showed enhanced activity of 24.65 U/mL (Figure 9.A). Different substrate conc. was incubated with L-asparaginase, among which the highest activity of the enzyme was achieved as 0.678 U/mL for 8 µg/mL concentration of substrate (Figure 9.B). The effect of

temperature on L-asparaginase activity is presented in Figure 9.C. The incubated enzyme with the substrate at various desirable temperatures yielded a maximum activity of 48.92 U/mL at 37°C.

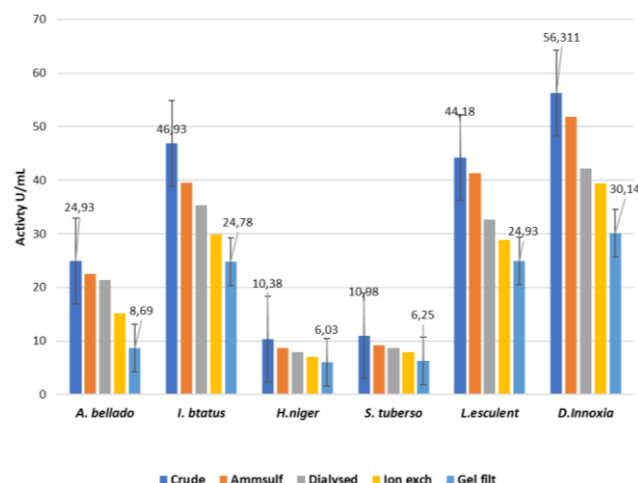


Figure 1. L-asparaginase enzyme activity in selected plant extracts at various purification steps

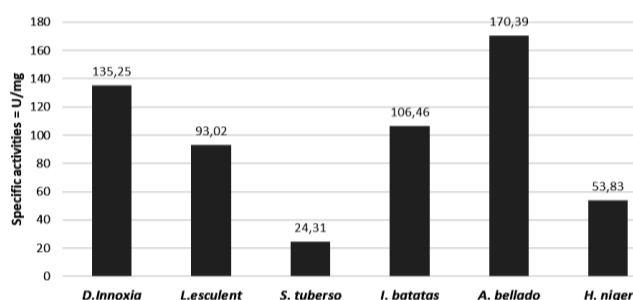


Figure 2. Specific activities of purified L-asparaginase from plant extracts

Table 1. L-asparaginase mean activity in selected plant extracts after purification (n=6)

Plant species	Activity of purified enzyme (mean) U/mL
<i>Datura innoxia</i>	30.14±0.928 ^A
<i>Lycopersicum esculentum</i>	24.93±0.538 ^B
<i>Ipomoea batatas</i>	24.78±0.743 ^B
<i>Atropa belladonna</i>	8.69±0.61 ^C
<i>Hyoscyamus niger</i>	6.03±0.493 ^D
<i>Solanum tuberosum</i>	6.25 ±0.344 ^{CD}

Note: Significant ($P<0.05$), ^{C, CD} Non-significant, ^A Significantly important, Means sharing the same letter are statistically non-significant

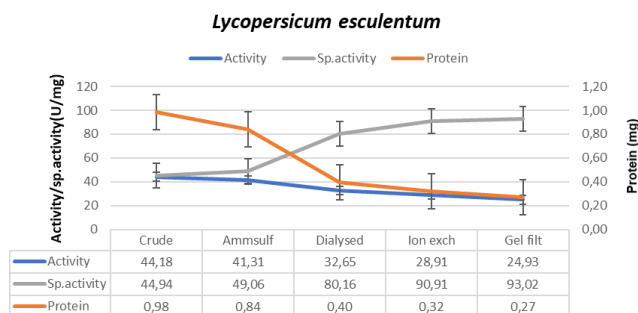


Figure 3. L-asparaginase activity, specific activity, and protein content during different purification steps from *L. esculentum*

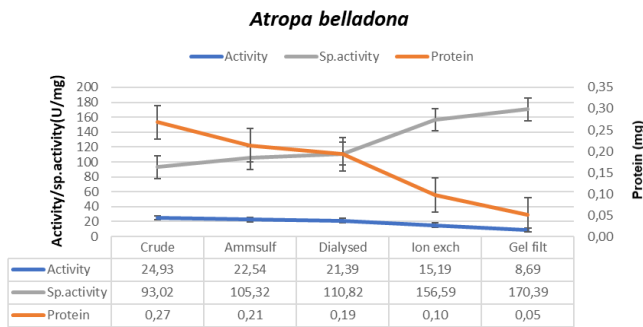


Figure 4. Enzyme L-asparaginase activity, specific activity, and protein content during different purification steps from *A. belladonna*

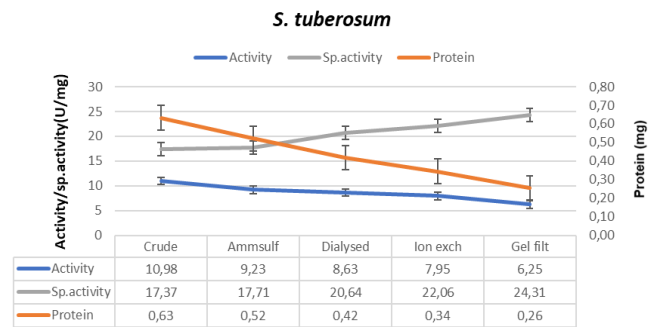


Figure 5. L-asparaginase activity, specific activity, and protein content during different purification steps from *S. tuberosum*

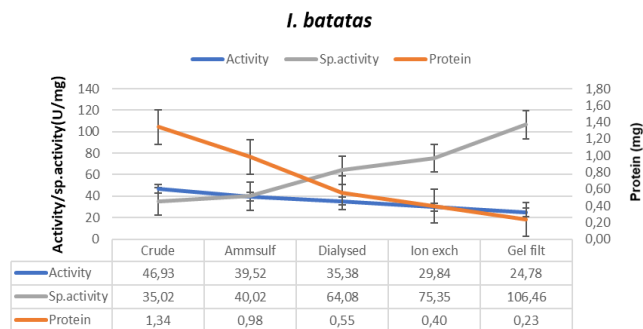


Figure 6. L-asparaginase activity, specific activity, and protein content during different purification steps from *I. batatas*

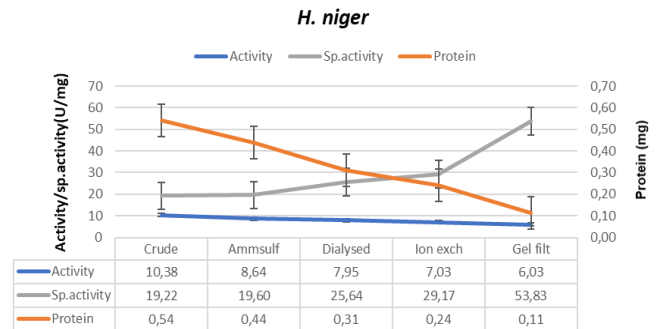
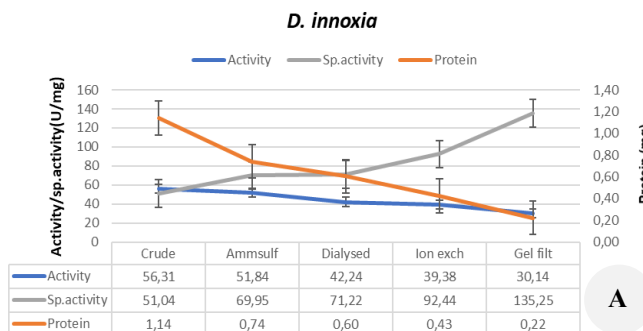
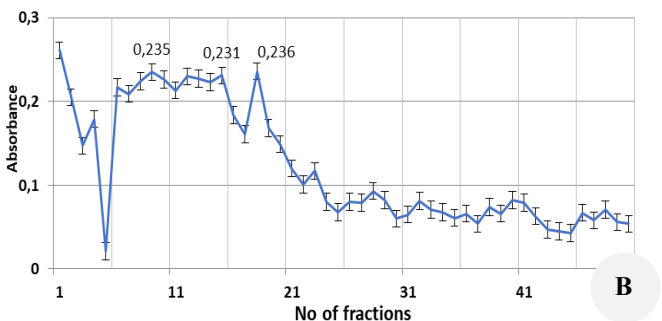


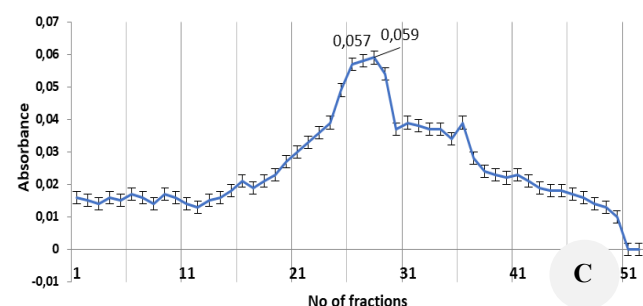
Figure 7. Enzyme L-asparaginase activity, specific activity, and protein content during different purification steps from *H. niger*



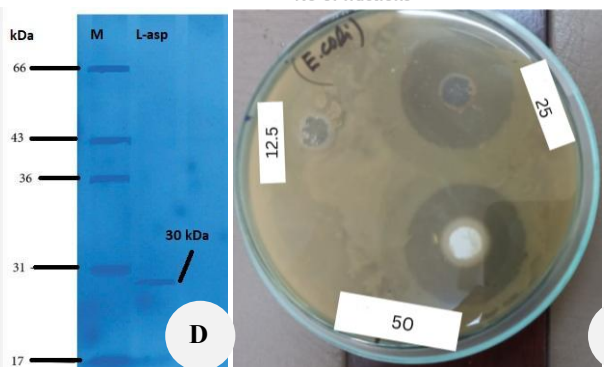
A



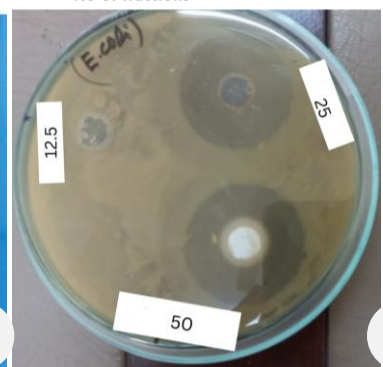
B



C



D



E

Figure 8. Elaborated purification summary and confirmation of purified L-asparaginase from *D. innoxia*. A. L-asparaginase activity, specific activity, and protein content during different purification steps; B. Ion exchange chromatography showed the highest protein content in fractions 9,15 and 19; C. Gel filtration chromatography resulted in enzyme concentration eluted in fractions no 25, 26, 27; D. SDS PAGE, L-asparaginase purified from *D. innoxia* determined by SDS PAGE; E. The minimal inhibitory concentration of E-extract of *D. innoxia* 6.25 mg/mL. Note: Lane M: Molecular weight marker proteins, L-asp: Coomassie blue stained fraction of purified enzyme protein after Gel filtration chromatography has molecular weight 30±1.5 kDa

Line weaver Burk enzyme kinetics of L-asparaginase from *D. innoxia*

Line weaver Burk plot shows a logarithmic increase of velocity and then a parabolic trend with maximum velocity reaching up to $V_{max} = 10.590$ $\mu\text{mol}/\text{min}$ and Michaelis constant $K_m = 0.1766$ μmol . Michaelis-Menton plot of enzyme L-asparaginase from *D. innoxia* depicted $1/V_{max} = 0.525$ $\text{min}/\mu\text{mol}$ and substrate concentration at which enzyme attained $\frac{1}{2} V_{max}$ ($K_m = 0.1766$ μmol) (Figures 10.A and 10.B). Both of the plots confirmed enzyme K_m as reconfirmed by www.physiology.com website for enzyme kinetics.

Antibacterial efficiency

Concentration gradient of the ethanolic extract 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mg/mL were employed to determine the MIC. The minimum inhibitory concentration of *D. innoxia* ethanolic extract (E- extract) was 6.25 mg/mL against microbial strains of *E. coli*, *S. aureus*, and *B. subtilis* (Table 2). The study found that the crude extract had bactericidal activity against the tested bacterial strain (Table 3). E- extract of *D. innoxia* had efficiently inhibited bacterial growth, i.e., 54.5, 33.30, and 45.65% in *E. coli*, *S. aureus*, and *B. subtilis*, respectively.

Anti-cancerous and antibacterial activity confirmation by bioinformatic tools

The function of L-asparaginase as an anticancer agent was verified using InterPro Scan, which identified its domain IPR027474 and active sites IPR020827 and IPR027475. These active sites catalyze L-asparagine deamination, leading to tumor cell death, as shown in

Figure 12. The Chimera docking results confirmed the interaction between chitin (bacterial cell wall component) and L-asparaginase with bond lengths of 2.32, 2.07, and 2.54 Å, an RMSD value of 0.00, and a score of -5.5, as depicted in Figure 11.A.

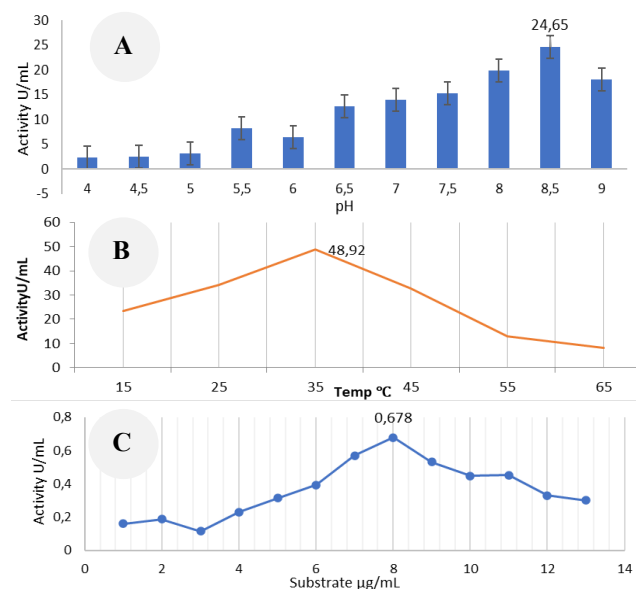


Figure 9. A. Effect of pH on the enzyme activity: pH 4-13; B. Temperature fluctuation from 15 to 65°C; C. Optimal substrate concentration on the activity of L-asparaginase in *D. innoxia* extract

Table 2. Minimum inhibitory concentration of *D. innoxia* ethanolic extract against microbial strains of *E. coli*, *S. aureus*, and *B. subtilis*

Plant extract	Minimum Inhibitory Concentration (mg/mL)						
Ethanolic extract	50	25	12.5	6.25	3.12	1.56	0.78
<i>E. coli</i>	-	-	-	-	*	*	*
<i>B. subtilis</i>	-	-	-	-	*	*	*
<i>S. aureus</i>	-	-	-	-	*	*	*

Note: -: No growth, *: Shows growth. Incubation temperature 37°C, Incubation time: 24h

Table 3. Antibacterial activity of *D. innoxia* ethanolic and aqueous extracts against microbial strains of *E. coli*, *S. aureus*, and *B. subtilis*

Extracts of <i>D. innoxia</i>	Microbial strains	Control growth (mm)	Treatment growth (mm)	Treatment growth (±SD)	% Inhibition of bacterial growth
Ethanol	<i>E. coli</i>	22	12.5	12 ±0.23	43.2
	<i>S. aureus</i>	18	6	6.0±0.8	66.7
	<i>B. subtilis</i>	23	10	10.5±1.4	56.5
Aqueous	<i>E. coli</i>	13	7	7.06±0.25	46.2
	<i>S. aureus</i>	11	4	4.01±0.61	63.6
	<i>B. subtilis</i>	14	6	6.02±0.49	57.1

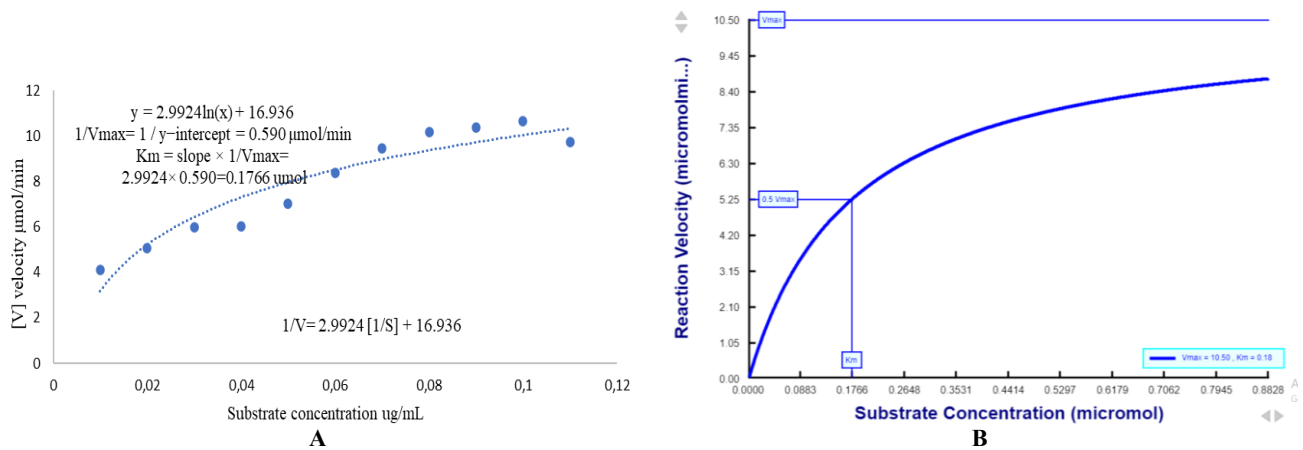
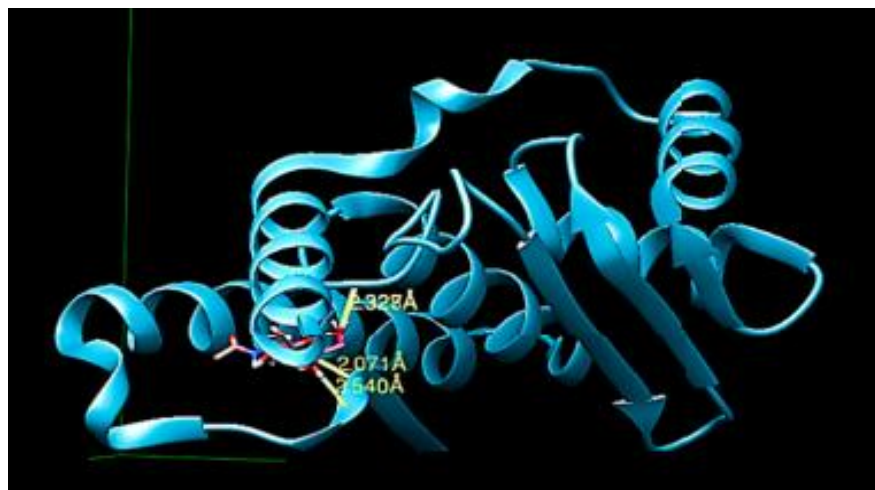
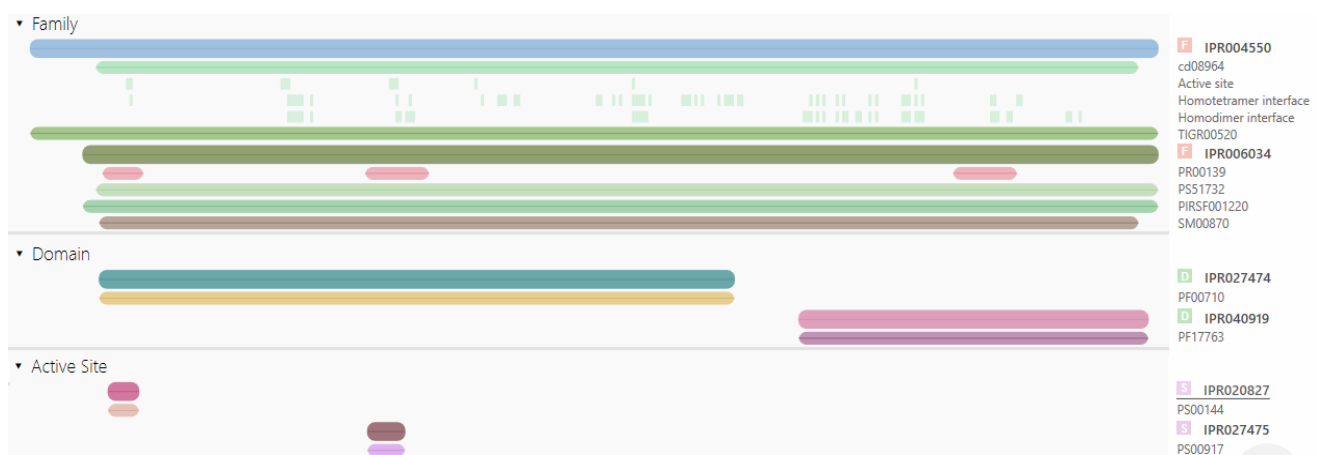


Figure 10. Enzyme kinetics of L-asparaginase as experimented from *D. innoxia*. A. Graph follows Line weaver burk enzyme kinetics, The logarithmic increase of velocity and then parabolic trend with $V_{\text{max}} = 10.590 \mu\text{mol}/\text{min}$ and $K_m = 0.1766 \mu\text{mol}$; B. Michaelis Menton plot of enzyme L-asparaginase from *D. innoxia*. with $1/V_{\text{max}} = 0.525 \mu\text{mol}/\text{min}$ and $K_m = 0.1766 \mu\text{mol}$. Note: Enzyme kinetics (Michaelis Menton constant and V_{max}) was plotted at www.physiologyweb.com



A



B

Figure 11. A. Chimera docking between chitin and L-asparaginase; B. L-asparaginase functional analysis to verify anticancer effectiveness. InterPro Scan validated L-asparaginase activity as an anticancer agent by exhibiting its domain IPR027474 and active sites IPR020827 and IPR027475

Discussion

L-asparaginase, also known as ASNase, plays a crucial role in cancer treatment to treat various types of cancer, including acute lymphocytic leukemia, lymphosarcoma, and melanoma sarcoma. The current biologically therapeutic protein is often administered with chemotherapeutic agents such as daunorubicin, cytosine arabinoside, vincristine, and L-asparaginase (Munir et al. 2019; Lee et al. 2021). In 2020, over 53,000 people were diagnosed with Acute Lymphoblastic Leukemia (ALL), and chemotherapy remains the standard treatment for ALL patients (Moharib 2018). L-asparaginase is an extracellular enzyme that acts as an asparagine amido hydrolase, breaking down asparagine (Asn) into ammonia and aspartic acid within cancer cells. Healthy cells can escape this shortage of asparagine by synthesizing Asn from L-asparaginase synthetase (Aisha et al. 2020b), which cancer cells lack. The commercially available L-asparaginase enzyme for clinical use is obtained from *Escherichia coli* and *Erwinia carotovora*; these sources can induce side effects and anaphylaxis (Effer et al. 2020). To mitigate these adverse reactions, researchers have turned to a phytomedicinal source to produce L-asparaginase for anticancer therapy. The focus of the study was to find the potent sources of L-asparaginase and their optimal milieu factors.

The objective of the research was to elucidate the highest enzyme activity exhibiting plants from the selected ones, which was characterized, and kinetics were revealed to provide insight into the novel pharmaceutical source. Solanaceae family possesses ample concentrations of L-asparaginase. The research conducted here included *D. innoxia*, *A. belladonna*, *H. niger* (leaves), *L. esculentum* (fruit), *S. tuberosum* (roots) and *Ipomoea batatas* (roots). All plants are latent sources of L-asparaginase containing variable concentrations, as shown in Figure 1. Purified enzymes from *D. innoxia* 30.14 U/mL exhibited the most significant activity, followed by *L. esculentum* and *Ipomoea batatas* 24.93 and 24.78 U/mL, respectively. L-asparaginase from *A. belladonna* has remarkable specific activity at 170.39 U/mg, followed by *D. innoxia*, *I. batatas*, and *L. esculentum* 135.25, 106.46, and 93.02 U/mg, respectively.

Among selected plants of the family Solanaceae, *D. innoxia* achieved the highest enzyme amount in crude and purified extracts. Enzyme sourced from the crude extract of *D. innoxia* exhibited activity 56.311 U/mL, protein amount 1.14 mg/mL and specific activity 51.04 U/mg as depicted in Figure 8.A. *Datura innoxia* provided 56.77% yield on purification, specific activity 30.14 ± 0.928 U/mL and activity 135.25 U/mg (Figure 8.A). *Datura*, a plant belonging to the Solanaceae family, is known to have ten species. Still, only two of them, namely *D. innoxia* and *Datura stromonium*, are known to have drug-like effects (Kirar et al. 2022). The plant has been reported to possess various properties, such as insect repellency, antioxidant activity, antimicrobial activity, anticancer potential, anti-inflammatory properties, and anticholinergic activity (Soni et al. 2012). Phytochemical analysis revealed alkaloids, steroids, glycosides, tannins, flavonoids, saponins, atropine, phenols, proteins, carbohydrates, and fats in *Datura*

(Sayyed and Shah 2014). *Datura innoxia*, also known as angel's trumpet, devil's trumpet, and other common names, is an herbaceous or sub-shrub plant that can grow up to 2 meters in height. It is considered an annual plant in dry environments but can behave as a perennial under favorable environmental conditions. *Datura* has been used in shamanistic rituals to achieve illumination and as a hallucinogenic agent by drug users. It has also been used in phytotherapeutic practices to treat conditions such as impotence, asthma, and diarrhea. However, it is important to highlight it is associated with adverse effects, including disorientation, amnesia, blurred vision, and even death in overdose cases (Matias et al. 2020).

While discussing the enzyme characterization and its milieu, our protein, i.e., L-asparaginase, worked best at a pH of 8.5 and temperature of 37°C, delivering the highest activity at a substrate conc. 27.54 U/mL. The researchers testified that the most suitable pH ranges between pH 7.0 and 8.5. However, its efficiency decreases at extremes of the pH scale. A separate investigation referenced in the present paper focusing on plants also revealed that the optimal pH for L-asparaginase activity lies between 8.2 and 8.5, comparable to the current study (Zobaify et al. 2016). It is essential to mention that the optimal pH for L-asparaginase might vary depending on the specific plant species or strain being studied. For *D. innoxia*, the optimal pH was 8.5 (Figure 9.A).

Researchers studied how well the enzyme L-asparaginase works at different amounts of its building blocks (substrates) and wanted to find the best level. They discovered that the enzyme works best when the substrate is 8 ug/mL in the solution. At this level, the enzyme showed an activity of 0.76 U/mL. This enzyme's activity was similar to enzymes from plants called *S. nigrum* (Mako) and *Capsicum annuum* (Chilli), which belong to the Solanaceae family (Aisha et al. 2020a, b, 2022). The enzymes from these plants also showed good activity at 66.27 and 112 U/mL, respectively. The amount of protein in the enzyme extract was 1.24 mg/mL; when compared with the enzymes from the two plants, the protein amounts and specific activities were 1.02 mg/mL and 90.15 U/mg for *S. nigrum* (Mako) and 1.02 mg/mL and 90.15 U/mg for *Capsicum annuum* (Chilli) (Aisha et al. 2020a,b, 2022). Interestingly, although many plants from the Solanaceae family have been studied, *D. innoxia* has yet to be reported to produce L-asparaginase until now. High enzyme activity is beneficial for treating childhood ALL (Acute Lymphoblastic Leukemia) by breaking down bodily substances (Khalaf 2012).

L-asparaginase works best at a temperature of 37°C. When the temperature is too high or too low, its effectiveness decreases. This matches the findings Khalaf et al. (2012). They discovered that enzymes generally have a specific temperature range in which they work best. Another study by Shanmuga Prakash and their team found that L-asparaginase remains stable up to 45°C (Figure 9.B). However, its activity is most stable at temperatures between 37 and 40°C, similar to the optimal temperature. In another experiment using L-asparaginase from *Pisum sativum*, the enzyme showed the highest activity at 37°C

(Figures 2.B and 2.C). They also found that phosphate is the best substance for stabilizing the enzyme (Shanmugaprakash et al. 2015).

According to Dixit et al. (2013), *D. innoxia* leaf tissue contains a maximum total protein content of 2.0905 ± 0.71 , so we chose leaves to extract the L-asparaginase enzyme (Dixit et al. 2013). L-asparaginase from *D. innoxia* exhibited an optimal temperature of 35.5°C , pH 8.5, with a substrate conc. of 8.5 ug/mL (Figure 8). At the same time, the enzyme characterized by *S. nigrum* had a slight variation in optimal profile, showing a temperature of 36.5°C , pH 8.4, and substrate conc. 8.0 ug/mL (Aisha et al. 2022). The molecular weight of $30 \pm 1.5 \text{ kDa}$ L-asparaginase elucidated from *D. innoxia* by SDS PAGE (Figure 8.A) is similar to research on *S. nigrum* had revealed L-asparaginase with a molecular weight of 32 kDa indicating that the molecular weight of the L-asparaginase molecule may vary even among plants.

MIC of *D. stramonium* extract in benzene was 0.78 mg/mL , as noted against *E. coli* and *S. aureus* (Table 2) (Baynesagne et al. 2017). According to Baynesagne et al. (2017), *D. stramonium* extract in chloroform showed the highest antibacterial activity against *S. aureus* (ATCC25923) 18.2 mm and the lowest antibacterial activity against *E. coli* (8.2 mm) (acetone extract). In the current study, the methanolic extract of *D. innoxia* showed a maximum $12 \pm 0.23 \text{ mm}$ zone of inhibition against *E. coli* followed by inhibition in microbial growth of *S. aureus* and *B. subtilis* 10.5 ± 1.4 , $6.0 \pm 0.8 \text{ mm}$, respectively as shown in Table 3 and Figure 8.E.

Chinese medicinal plants have been found to possess diuretic and antipyretic properties which help to alleviate inflammations like edema. The methanolic extract of *D. innoxia* cures asthma and gastric issues and is also used in antitumor treatment. Antitumor assays were conducted to ascertain the enzyme efficiency against microbes and cancer cells (Monteiro et al. 2014; Noor et al. 2014, 2016; Tohyama et al. 2014). As reported by Sharma et al. (2021), *Solanum surattense* crude extract has proven remarkable anticancer activity at 76% in a cytotoxicity assay with MIC 15 mg/mL and antitumor activity establishing, i.e., 75% inhibition of tumor (Sharma et al. 2021). L-asparaginase also exhibits antioxidant and antiproliferative properties and is regarded as an anti-cancerous biomolecule (Aisha et al. 2022). Owing to its mechanism, L-asparaginase transforms asparagine into aspartic acid (Batool et al. 2016), whereas insufficient levels of the enzyme asparagine synthetase are seen in cancer cells. Therefore, asparagine depletion leading to cell death occurs because the conversion from asparagine to aspartic acid is irreversible (Alrumman et al. 2019). The specific domains of our protein and active sites trigger L-asparagine deamination. To verify this claim, the estimation of function prediction confirmed domains and active sites of asparaginase (Figure 11), catalyzing L-asparagine deamination into aspartic acid and ammonium ions (Chiu et al. 2019). Chimera docking showed significant interactions between chitin and L-asparaginase, with bond lengths of 2.32, 2.07, and $2.54 \text{ \AA}$, an RMSD value of 00, and a score of 5.5 (Figure 11).

InterPro Scan identified the curative properties of L-asparaginase containing subdomain IPR027474 and active sites IPR020827 and IPR027475. The active sites accelerate the deamination of L-asparagine, leading to the death of tumor cells. Figure 11 shows a specific case of functional analysis. The ideal outcomes featured the maximum number of hydrogen bonds, the lowest energy score, and the RMSD value around zero. The ideal docking findings involved maximum hydrogen bonds with lengths of RMSD value 0.00 with a score of -5.5. The study concluded that L-asparaginase and the chitin monomer N-acetylglucosamine formed a successful connection.

As a result, our plant extracts encompassed a substantial amount of L-asparaginase. In addition, the most effective extract considerably reduced microbial and tumor development. The results are consistent with those of other plant-origin enzymes for which pure L-asparaginase has been reported.

In conclusion, extracts of various plants of the Solanaceae family, particularly leaves of *D. innoxia*, possess a high titer of L-asparaginase. Moreover, its bactericidal efficiency also makes it a suitable candidate for novel pharmaceuticals. The contents of L-asparaginase in *D. innoxia* extract are sufficient and thus can be utilized in chemotherapy, as L-asparaginase mitigates antitumor action by its two main active sites.

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Therapeutic potential of combined *Gongronema latifolium* fruit and leaf extracts against oxidative stress and organ damage

CHIAMAKA VIVIAN OKOCHI^{1,*}, S. C. UDEDI¹, K. K. ASOGWA¹, E. J. OTOVBO², M. S. OBI¹,
U. A. EZEAKU¹

¹Department of Applied Biochemistry, Nnamdi Azikiwe University. Along Enugu-Onitsha Expressway, Ifite Road, Awka 420110, Anambra, Nigeria.
Tel.: +234-8132743397, *email: Chiamakaokochi808@gmail.com

²Department of Biochemistry, Dennis Osadebay University. Along Asaba-Benin Expressway, Anwai Road, Asaba 320242, Delta State, Nigeria

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Abstract. Okochi CV, Udedi SC, Asogwa KK, Otoybo EJ, Obi MS, Ezeaku UA. 2025. Therapeutic potential of combined *Gongronema latifolium* fruit and leaf extracts against oxidative stress and organ damage. *Asian J Nat Prod Biochem* 23: 38-46. This study aims to evaluate the therapeutic potential of combined extracts of the fruit and leaves of *Gongronema latifolium* in managing oxidative stress and protecting against liver and kidney damage. Forty albino rats were randomly assigned to eight groups (A-H), each consisting of 5 rats. Groups A-C served as the control groups, with Group B as the positive control group, receiving silymarin. Groups D-H were administered different ratios of *G. latifolium* fruit and leaf extracts at 100 mg/kg BW. The biochemical analysis and histological study of the liver were conducted using standard procedures. Results from the acute toxicity study showed that the LD₅₀ value of the extract was >5000 mg/kg BW, categorized as relatively harmless; the combined extracts significantly increased antioxidant enzyme activities (CAT, GSH, SOD) and reduced malondialdehyde (MDA) levels. Most combined extracts demonstrated anti-hepatotoxic effects by lowering ALT, AST, and ALP levels. The combined extracts of fruit and leaf of *G. latifolium* demonstrated potential for renal protection, as evidenced by reduced urea and creatinine levels, particularly in groups receiving a higher ratio of fruit extract. Histological analysis showed normal liver tissue, indicating no adverse effects from the extracts. In conclusion, these combinations of fruit and leaf extracts of *G. latifolium* could be natural antioxidants and protective agents against environmental toxins, with possible applications in traditional medicine and therapeutic strategies.

Keywords: Anti-hepatotoxicity, anti-nephrotoxicity, antioxidant, drug discovery, *Gongronema latifolium*, oxidative stress

INTRODUCTION

WHO (2023) stated that approximately 80% of the world's population uses traditional medicine for primary healthcare. It supports research showing that healthcare globally has historically relied heavily on traditional medical systems for their potential therapeutic and medicinal benefits (Aladejana 2023). In recent years, there has been a growing global interest in the toxicity associated with environmental pollutants, the consumption of highly processed food, and counterfeit drinks, which can accumulate in the body for a long time, leading to oxidative stress, inflammation, and organ damage (Sokan-Adeaga et al. 2023). There is a need to investigate alternative therapies to combat this detrimental process by exploring natural plant products. Oxidative stress has been recognized as a deleterious process associated with various health issues, including cell damage, autoimmune disorders, aging, cancer, cardiovascular disease, and degenerative diseases (Asogwa et al. 2020).

Naphthalene, a white, crystalline Polycyclic Aromatic Hydrocarbon (PAH) with the formula C₁₀H₈, comprising two fused benzene rings, exhibits high melting and boiling points. Its aromatic character makes it susceptible to various reactions, including electrophilic aromatic substitution, oxidation, and reduction, leading to valuable derivatives (Giri and Moon 2024), such as 1-methylnaphthalene (1-

MN) and 2-methylnaphthalene (2-MN), which are the most abundant airborne PAHs (Chen et al. 2016; Fang et al. 2021), primarily emitted from biomass burning, combustion of fossil fuels, and industrial sectors (Fang et al. 2021). A study by Ye et al. (2024) revealed the significance of naphthalene and its derivatives contributing to the formation of Secondary Organic Aerosol (SOA) and other secondary pollutants in the Yangtze River Delta (YRD) region during summer, which may pose environmental risks and result in adverse health effects. On the other hand, silymarin is a natural compound derived from *Silybum marianum* (L.) Gaertn. (milk thistle) that has been well-researched for liver diseases (Abenavoli and Milic 2017). It's an isomeric combination of flavonolignans, exhibiting inherent hepatoprotective and antioxidant activity by controlling free radicals produced during the hepatic metabolism of toxic chemicals like ethanol, acetaminophen, or carbon tetrachloride (Qadir and Sahoo 2021).

Gongronema latifolium NIF 2345 is a plant that belongs to the family Asclepiadaceae with significant nutritional and medicinal properties, particularly in managing diabetes and oxidative stress, as shown in recent studies on its leaf extract's antidiabetic and antioxidant effects (Ojo et al. 2020). It is also a tropical rainforest plant whose leaves have a characteristic sharp, bitter, and slightly sweet taste, especially when eaten fresh (Omodale et al. 2017). In comparison, its fruit has smooth, greenish skin

when unripe, which then turns brown and black at maturity (Okochi et al. 2024). Their common names in Nigeria are bushbuck in English, *utazi* in Igbo, *madumaro* or *aroکه* in Yoruba, and *utasi* among the Ibibios and Efik (Elijah et al. 2022). Different parts of *G. latifolium* NIF 2345, including leaves, fruit, root, and stem, contain distinct chemical substances that can exert specific physiological effects, making them valuable sources of therapeutic agents (Parsaeimehr et al. 2017). It is used for culinary practice, such as vegetables in soups and salads, or spices in different food preparations (Amrelia et al. 2022), and polyherbal decoction for hepatitis and malaria in traditional medicines because it helps in cleansing the liver (Ihesie 2022), alleviating symptoms of catarrh, congestion, and cough (Juliani et al. 2013), treating hyperglycemia (Ogunyemi et al. 2020), stomach ache, laxative (Osuagwu et al. 2023), and fruit preservative (Ejembi et al. 2022). Previous research on *G. latifolium* NIF 2345 fruit and leaves exhibits good nutritive value, anti-helminthic activity, and anti-malarial activity (Orumwensodia and Uadia 2022; Ojo et al. 2023), anti-bacterial, anti-fungal, and anti-oxidative properties (Onwukeme et al. 2023; Bassey et al. 2020).

Limited research has been conducted on the specific impact of *G. latifolium* NIF 2345 leaf and fruit extracts, or their combination, on naphthalene-induced oxidative stress in the liver and kidneys. This study aims to investigate the natural antioxidant, anti-nephrotoxicity, and hepatoprotective properties of combined *G. latifolium* NIF 2345 leaf and fruit extracts in mitigating naphthalene-induced oxidative stress. The present study investigates the effects of combined *G. latifolium* NIF 2345 fruit and leaf extracts on naphthalene-induced oxidative stress in rats at 100 mg/kg. This study was conducted to develop innovative therapeutic approaches for oxidative stress-related health issues, provide scientific validation for the traditional use of *G. latifolium* NIF 2345, and promote the inclusion of *G. latifolium* fruit in medicinal and dietary applications.

MATERIALS AND METHODS

Materials

Fruit and leaf samples of *G. latifolium* NIF 2345 were collected from Okochi's compound in Umudunu Village, Abagana, Njikoka L.G.A., Anambra State, Nigeria. The plant samples were sent to the Department of Botany Herbarium at Nnamdi Azikiwe University, Awka, for identification and authentication by Mr. Iroka Chisom, a department taxonomist. A voucher specimen was deposited with voucher numbers NAUH-34D and NAUH-34A.

Study area

This study was conducted at the Natural Products Laboratory, Unizik Awka, Animal House Department of Applied Biochemistry, Nnamdi Azikiwe University, Awka, and Ifex's Diagnostics laboratory, Regina Caeli Road, Awka.

Duration

The study was completed in 70 days, equivalent to 10 weeks, including 14 days for air-drying, 5 days for

pulverization and extraction, 7 days for acclimatization, 30 days for animal studies, and 14 days for in vivo assays, data collection, and data analysis.

Procedures

One kilogram (1 kg) of the pulverized *G. latifolium* NIF 2345 leaves and fruits was soaked separately in 10 L of deionized water and left for 24 hours. After 24 hours, the sample was filtered and concentrated at 60°C using a water bath (Memmert WTB). The crude extracts were transferred into a sample bottle, stored at 4°C in the refrigerator, and used for further laboratory test analysis.

$$\text{Percentage of extract yield} = \frac{\text{Weight of extract}}{\text{Weight of dried homogenized sample}} \times 100\%$$

(Barros et al. 2008)

Animal studies

Ethical approval for all experimental protocols was obtained from the Animal Research Ethics Committee (AREC), Nnamdi Azikiwe University, Awka, with the NAU/AREC/2024/0081 reference number. This research study followed the guidelines for laboratory animal use and care provided in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) and the US guidelines (NIH publication #85-23, revised in 1985).

Purchase, acclimatization, and feeding of animals

The experiment followed a completely randomized design. Forty (40) albino rats were purchased from Onyewuchi Farm, Ifite, Awka. These animals were acclimatized for one week and fed only food and water.

Determination of LD₅₀ of extract

The acute toxicity study used Lorke's method (Lorke 1983). In the first phase, six rats were randomly divided into three groups (n = 1 rat/group). The groups received oral doses of 10, 100, and 1,000 mg/kg BW of the extract via a cannula. The rats were monitored for 24 hours for adverse effects and mortality. In the second phase, the procedure was repeated using six rats randomly divided into three groups (n = 1 rat/group). The groups received oral doses of 1,600, 2,900, and 5,000 mg/kg BW of the extract. The rats were observed for signs of mortality and toxicity effects.

Induction of oxidative stress

Oxidative stress was induced using the method of Vijayavel et al. (2007) by administering 1,100 mg/kg of naphthalene to the experimental animals and monitoring the results within 24 hours after naphthalene administration. The level of oxidative stress was determined by analyzing the concentration of malondialdehyde in the serum.

Body weight of experimental animal

The weight of the experimental subjects was measured using an electronic weighing scale. The body weight was monitored before, during, and after the experiment.

Grouping of the animal

Forty (40) albino rats were divided into eight (8) groups of five (5) animals in each group. They were grouped as

follows: Group A-normal control; Group B-positive control (induced and treated with silymarin); Group C-negative control (induced but not treated); while Groups D, E, F, G, and H were induced with naphthalene and treated with fruit and leaf extract at different ratios of 100 mg/kg; where Group D-(20 leaf + 80 fruit); Group E-(40 leaf + 60 fruit); Group F-(50 leaf + 50 fruit); Group H-(80 leaf + 20 fruit). The study was carried out for 28 days.

Blood collection

At the end of the experiment, the animals were anesthetized with chloroform. The blood was collected in a plain bottle via cardiac puncture and then centrifuged for 15 minutes at 4,000 rpm. The serum was collected and used for further analyses.

In vivo antioxidant activity

The effect of the combined fruit and leaf extract ratio was analyzed for antioxidant enzymes. Malondialdehyde was determined using the spectrophotometric method at 532 nm following the procedure of Buege and Aust (1978); reduced glutathione level was determined at 412 nm according to the method of Exner et al. (2000). Superoxide dismutase activity was determined by the increase in absorbance at 480 nm described by the method of Sun and Zigma (1978). Catalase activity was measured by the increase in absorbance at 620 nm, as described by Sinha's method (Sinha 1972).

Kidney function test

Urea and creatinine were analyzed using Randox test kits. The procedures followed the manufacturer's instructions.

Liver function test

Serum biochemical indices routinely estimated for liver functions, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total protein, were determined using Randox diagnostic kits. The procedures followed the manufacturer's instructions.

Histological analysis

Histopathological evaluations of the liver were carried out after the completion of the 4-week experimental procedure. The experimental animal was sacrificed by diethyl ether inhalation, and the abdominal cavity was exposed. Thereafter, liver tissues were processed for light histological microscopy, and conventional histopathological evaluations of the liver were conducted using the hematoxylin-eosin technique, as previously described by Akinlolu et al. (2017).

Data analysis

The data were expressed as mean $\pm$ Standard Error of the Mean (SEM) and subjected to One-Way Analysis of Variance analysis (ANOVA). Values were considered statistically significant at ($p < 0.05$) using Statistical Package

for Social Sciences (SPSS) software version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Percentage yield of extract

The percentage yields of the crude extract of *G. latifolium* NIF 2345 fruit and leaf were 15.3 and 15.0%, respectively.

Acute toxicity study via oral administration

The results of the acute toxicity study revealed no observable signs of toxicity at doses ranging from low to high (10-5,000 mg/kg) within the first 24 hours of exposure, as shown in Table 1.

Result on mean-weight parameters

Body weights of the rats were recorded on day 0 (before the acclimatization period), day 7 (before the induction of naphthalene), day 9 (24 hours after naphthalene administration), day 18 (after 9 days of treatment), and day 28 (after 18 days of treatment) (Table 2). The induction of naphthalene showed a significant difference in the body weight of the rats between the negative control, positive, and normal control groups. The body weight was increased significantly ($p < 0.05$) in the normal group (A) and positive control (B) on days 18 and 28 compared to days 0 and 7. The body weight in group D (20 leaf: 80 fruit mg/kg) and F (50 leaf: 50 fruit mg/kg) was significantly higher than that of the normal and negative control groups at day 28. The body weight of other treatment groups exhibited no significant difference compared to normal and positive control groups.

In vivo antioxidant enzyme activity

MDA, CAT, SOD, and reduced GSH activity of the experimental animals were assayed to determine the effect of the administration of a combination extract of *G. latifolium* NIF 2345 on these antioxidant enzymes. The results reveal no significant difference ($P > 0.05$) among the treated groups (D to H) when compared to the control groups (A-C), as seen in Table 3 and Figure 1.

Table 1. Signs of toxicity and mortality rate of rats treated with aqueous extract of *Gongronema latifolium* NIF 2345 fruit and leaf

Extract dose (mg/kg)	<i>G. latifolium</i> leaf		<i>G. latifolium</i> fruit	
	Observation	Mortality	Observation	Mortality
10	Normal	0/1	Normal	0/1
100	Normal	0/1	Normal	0/1
1,000	Normal	0/1	Normal	0/1
1,600	Normal	0/1	Normal	0/1
2,900	Normal	0/1	Normal	0/1
5,000	Slightly weak	0/1	Normal	0/1

Table 2. The effect of treatment on the body weight (g) of experimental rats

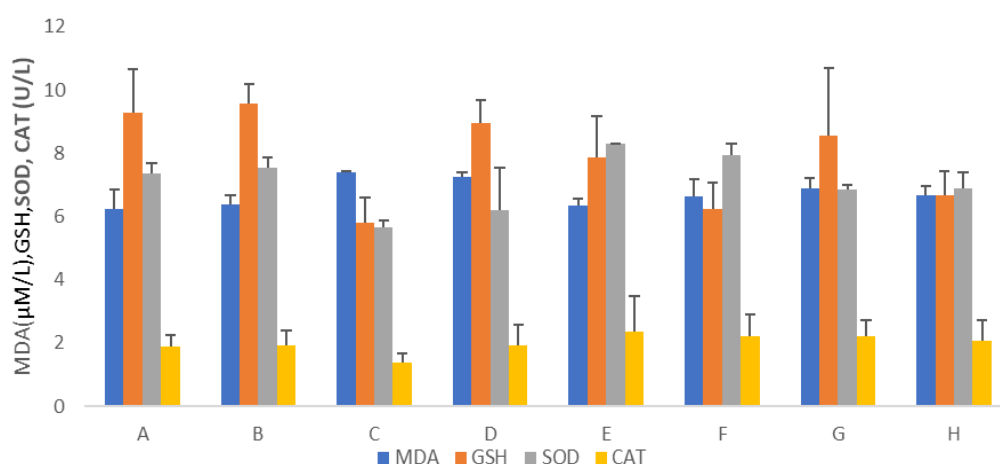
Groups (leaf:fruit)	Body weight (mg)				
	0 day	7 days	9 days	18 days	28 days
A (normal control)	113.50±2.90	116.50±2.90	117.75±3.09	119.75±5.38	122.25±3.09
B (positive control)	108.00±3.93	110.00±3.93	106.75±4.11	112.50±3.30	117.00±3.10
C (negative control)	108.00±5.35	110.25±5.23	105.25±5.12	102.5±4.94	98.25±4.87
D (20:80)	114.5±2.96 ^{bdf}	117.25±2.76 ^{bdf}	114.75±2.85 ^{bde}	119.75±2.55 ^{bde}	125.25±2.40 ^{ace}
E (40:60)	113.00±3.58 ^{bdf}	116.25±3.56 ^{bdf}	114.25±3.32 ^{bde}	116.25±3.32 ^{bde}	122.25±1.93 ^{bde}
F (50:50)	115.25±1.79 ^{bdf}	118.25±1.79 ^{bdf}	115.25±1.79 ^{bde}	120.25±1.79 ^{bde}	124.00±2.16 ^{ace}
G (60:40)	113.75±5.32 ^{bdf}	116.50±5.37 ^{bdf}	111.28±6.65 ^{bde}	114.00±6.72 ^{bde}	113.75±5.32 ^{bde}
H (80:20)	116.50±4.51 ^{bdf}	118.75±4.41 ^{bdf}	110.50±4.41 ^{bde}	114.00±4.60 ^{bde}	116.50±4.51 ^{bde}

Note: Each value represents the Mean ±SEM of five rats per group. ^aSignificant difference compared to normal control, ^bNo significant difference compared to normal control, ^cSignificant difference compared to positive control, ^dNo Significant difference compared to positive control, ^eSignificant difference when compared to negative control, ^fNo Significant difference when compared to negative control

Table 3. In vivo antioxidant enzyme activity of rats treated with various combinations of leaf and fruit extract of *Gongronema latifolium* NIF 2345 extracts

Groups (leaf:fruit)	MDA±SEM (μmol/L)	GSH±SEM (IU/L)	SOD±SEM (IU/L)	CAT±SEM (IU/L)
A (normal control)	6.25±0.59	9.29±1.38	7.35±0.32	1.90±0.34
B (positive control (silymarin))	6.38±0.28	9.56±0.63	7.55±0.33	1.92±0.46
C (negative control)	7.40±0.03	5.81±0.79	5.66±0.23	1.36±0.29
D (20:80 mg/kg)	7.26±0.14 ^{bdf}	8.97±0.69 ^{bdf}	6.18±1.37 ^{bdf}	1.91±0.68 ^{bdf}
E (40:60 mg/kg)	6.36±0.19 ^{bdf}	7.85±1.32 ^{bdf}	8.29±0.00 ^{bdf}	2.36±1.11 ^{bdf}
F (50:50 mg/kg)	6.62±0.56 ^{bdf}	6.25±0.1 ^{bdf}	7.92±0.37 ^{bdf}	2.22±0.69 ^{bdf}
G (60:40 mg/kg)	6.87±0.36 ^{bdf}	8.56±2.14 ^{bdf}	6.86±0.13 ^{bdf}	2.21±0.51 ^{bdf}
H (80:20 mg/kg)	6.68±0.29 ^{bdf}	6.78±0.76 ^{bdf}	6.89±0.50 ^{bdf}	2.08±0.65 ^{bdf}

Note: Each value represents the Mean ±SEM of five rats per group. ^bNo significant difference when compared to normal control, ^dNo significant difference when compared to positive control, and ^fNo significant difference when compared to negative control

**Figure 1.** Effect of combined *Gongronema latifolium* fruit and leaf NIF 2345 extract on in vivo antioxidant enzyme activity

Liver analysis results

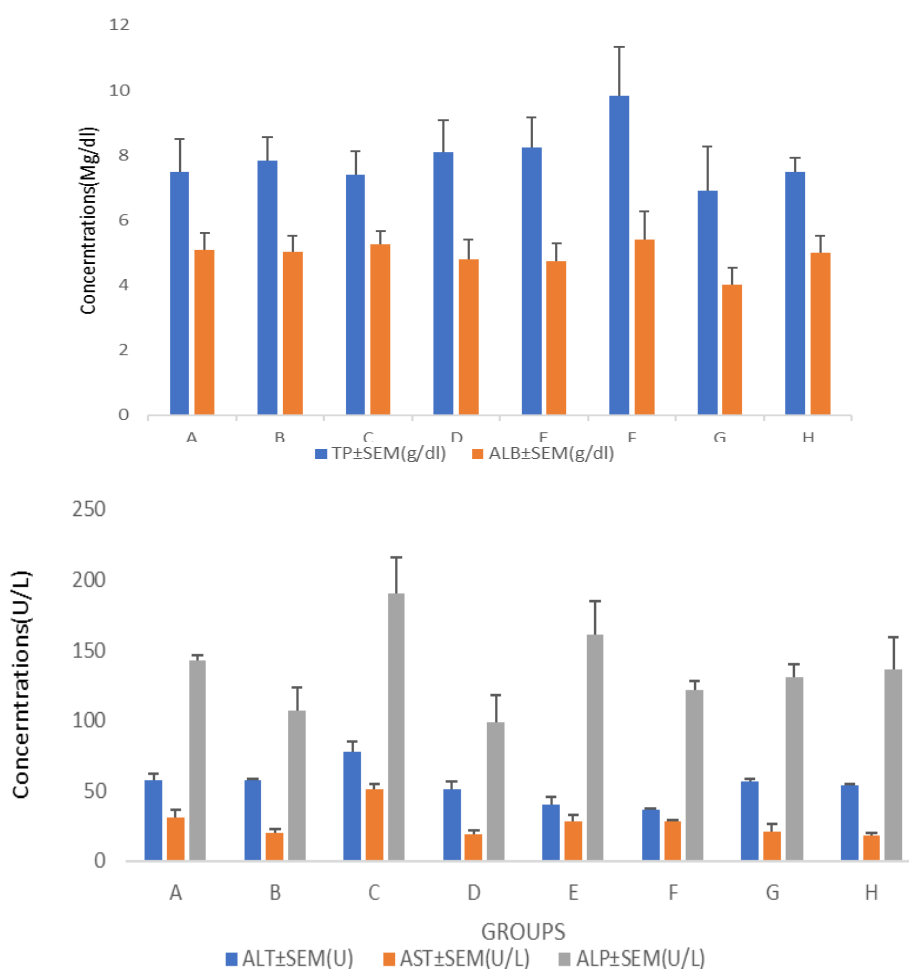
The induction of naphthalene results in elevated liver function parameters (ALP, AST, ALB, TP, and ALT). The results showed that there was no significant increase ($p < 0.05$) in the levels of AST and ALT among all groups (D to H) treated with *G. latifolium* extracts compared to the normal and positive controls. However, they indicated a significant reduction compared to the negative control. There were no notable changes in ALP, TP, and ALB

levels among the groups receiving combined *G. latifolium* fruit and leaf extract compared to the normal, positive, and negative controls ($P > 0.05$). It suggests that the combination of *G. latifolium* fruit NIF 2345 and leaf extract does not significantly impact ALP, TP, and ALB levels compared to the controls. Therefore, these specific treatments may not significantly alter the liver function parameters, as presented in Table 4 and Figure 2.

Table 4. Effects of the treatment of combined *Gongronema latifolium* NIF 2345 fruit and leaf extract on the liver function parameters

Groups (leaf:fruit)	ALT±SEM (U)	AST±SEM (U/L)	ALP±SEM (U/L)	TP±SEM (g/dl)	ALB±SEM (g/dl)
A (normal control)	57.60±4.14	30.49±5.89	143.05±3.61	7.50±1.01	5.08±0.54
B (positive control (silymarin))	57.82±0.59	19.90±2.29	107.16±16.17	7.82±0.75	5.04±0.49
C (negative control)	77.52±7.19	51.12±3.34	190.39±25.36	7.39±0.72	5.25±0.42
D (20:80 mg/kg)	50.61±6.37 ^{bde}	18.86±2.89 ^{bde}	98.74±19.53 ^{bdf}	8.08±1.00 ^{bdf}	4.80±0.60 ^{bdf}
E (40:60 mg/kg)	40.19±4.95 ^{bde}	27.72±4.54 ^{bde}	160.73±24.05 ^{bdf}	8.23±0.93 ^{bdf}	4.75±0.55 ^{bdf}
F (50:50 mg/kg)	35.93±1.26 ^{ace}	27.82±1.40 ^{bde}	121.32±6.80 ^{bdf}	9.84±1.50 ^{bdf}	5.41±0.85 ^{bdf}
G (60:40 mg/kg)	56.68±1.31 ^{bde}	20.42±6.04 ^{bde}	130.69±9.32 ^{bdf}	6.91±1.37 ^{bdf}	4.03±0.50 ^{bdf}
H (80:20 mg/kg)	53.65±1.14 ^{bde}	17.65±2.34 ^{bde}	136.16±23.00 ^{bdf}	7.49±0.43 ^{bdf}	5.01±0.50 ^{bdf}

Note: Each value represents the Mean ± SEM of four rats per group. ^bNo significant difference compared to normal control, ^dNo significant difference compared to positive control, ^eSignificant difference compared to negative control, ^fNo significant difference compared to negative control

**Figure 2.** Liver parameters of the group treated with combined *Gongronema latifolium* NIF 2345 extract at 100 mg/kg

The effect of administration of *Gongronema latifolium* leaf and fruit extract on the parameter of kidney function

The effects of *G. latifolium* leaf and fruit extracts on kidney function showed that all groups treated with *G. latifolia* showed significant reductions in urea and creatinine levels ($p < 0.05$) compared to the negative control group. The treated groups exhibited kidney function parameters comparable to the positive control group (silymarin). These

findings suggest that the leaf and fruit extracts may possess kidney protective properties, as shown in Figure 3 and Table 5.

Results of histology studies of the liver

The following photomicrographs were taken from the various treatment groups to present the effect(s) of *G. latifolium* NIF 2345 on the liver.

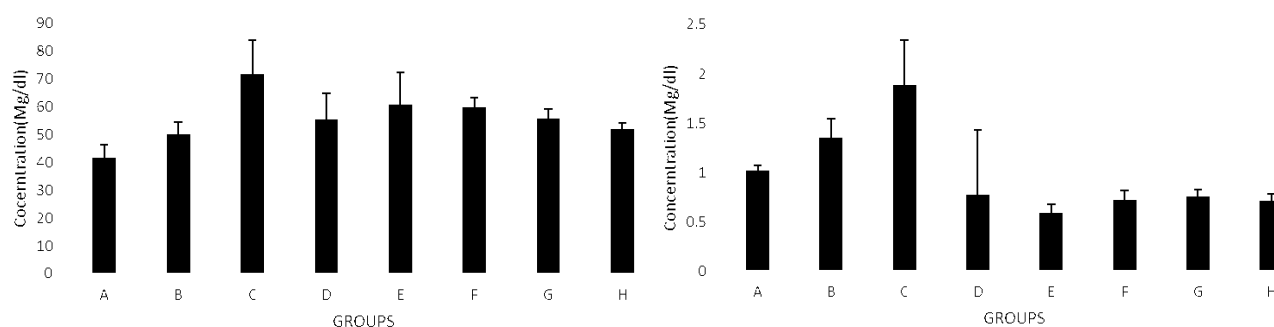


Figure 3. Effect of combined *Gongronema latifolium* fruit and leaf aqueous extract on kidney function parameters

Table 5. Effect of the treatment of combined *Gongronema latifolium* NIF 2345 fruit and leaf on kidney function

Groups (leaf:fruit)	Urea $\pm$ SEM (mg/dL)	Creatinine $\pm$ SEM (mg/dL)
A (normal control)	41.74 $\pm$ 4.63	1.02 $\pm$ 0.05
B (positive control (silymarin))	50.26 $\pm$ 4.34	1.35 $\pm$ 0.19
C (negative control)	71.63 $\pm$ 12.46	1.89 $\pm$ 0.45
D (20:80 mg/kg)	55.35 $\pm$ 9.40 ^{bdf}	0.77 $\pm$ 0.66 ^{bde}
E (40:60 mg/kg)	60.83 $\pm$ 11.65 ^{bdf}	0.59 $\pm$ 0.09 ^{bde}
F (50:50 mg/kg)	59.74 $\pm$ 3.72 ^{bdf}	0.72 $\pm$ 0.10 ^{bde}
G (60:40 mg/kg)	55.9 $\pm$ 3.32 ^{bdf}	0.76 $\pm$ 0.07 ^{bde}
H (80:20 mg/kg)	51.90 $\pm$ 2.28 ^{bdf}	0.71 $\pm$ 0.07 ^{bde}

Note: Each value represents the Mean $\pm$ SEM of four rats per group.

^aSignificant difference compared to normal control, ^bNo significant difference compared to normal control, ^cSignificant difference compared to positive control, ^dNo significant difference compared to positive control, ^eSignificant difference when compared to negative control, ^fNo significant difference when compared to negative control

Discussion

Medicinal plants have been used for centuries to prevent and treat diseases, with much of the world's population still relying on traditional or herbal medicine across cultures and continents (Fitzgerald et al. 2020). Phytochemicals are bioactive components in traditional herbal medicines and are formulated in herbal formulations (Tanaka and Kashiwada 2022). An environmental toxicant, naphthalene, has been reported to induce oxidative stress through inhalation, ingestion, and dermal contact, leading to hemolytic anemia, liver damage, retinal damage, and neurological issues (Fang et al. 2021). On the other hand, silymarin is a synthetic drug known for its antioxidant and anti-hepatotoxicity properties (Abenavoli and Milic 2017).

Previous research on *G. latifolium* NIF 2345 shows that *G. latifolium* exhibits analgesic, antitumor, broad-spectrum antimicrobial, and anti-sickling (Damunupola et al. 2014) and may offer protection against hematological complications associated with diabetes and toxic environmental exposures (Effiong et al. 2024). The Lethal Dose (LD50) assessment in measuring acute toxicity and using fewer animals in acute toxicity studies are very important for ethical reasons

(Chinedu et al. 2013). The findings showed that the median lethal dose of *G. latifolium* NIF 2345 fruit and leaf extract was $\geq 5,000$ mg/kg, categorized as relatively harmless. Administration of aqueous extracts of combined *G. latifolium* NIF 2345 reduced the weight-altering effects of naphthalene (1,100 mg/kg) in rats without significant changes in body and organ weights compared to positive and normal controls. This finding supports previous research on the nutritional value and potential health benefits of *G. latifolium* NIF 2345 (Nneoyi-Egbe et al. 2024).

Antioxidants are the compounds in charge of scavenging free radicals and protecting our bodies from a wide range of diseases associated with free radicals (Alam et al. 2020). The results of our study (Table 3; Figure 1) showed no significant reduction ($p < 0.05$) in the in vivo antioxidant enzyme of the rat groups treated with naphthalene and administered with the combined extract. It revealed that the combined extract exhibits good antioxidant potential. However, group E (40% leaf:60% fruit mg/kg) had lower MDA levels and higher GSH, SOD, and CAT levels compared to other experimental groups. It closely aligns with Analike et al. (2022) findings on the antioxidant potential of an aqueous extract of *G. latifolium* NIF 2345 leaves, which reduced heart malondialdehyde levels and increased antioxidant enzyme levels. The presence of phytochemicals in *G. latifolium* NIF 2345 fruit and leaf, such as phenols, saponins, and tannins, contributed to the antioxidant effects to prevent oxidative damage by scavenging reactive oxygen species (Okochi et al. 2024).

Numerous studies have demonstrated the hepatoprotective benefits of silymarin (Eze et al. 2021). The findings of this study showed that silymarin treatment reduced AST and ALP levels compared to the negative control group (group C) but not to the normal control group (group A). Group D (20% leaf:80% fruit mg/kg) showed a significant decrease in ALT levels compared to the normal and negative control groups. It might relate to the findings of Adeyemi-Doro et al. (2021) that *G. latifolium* decreased liver enzyme activities, indicating the hepatoprotective effect. Previous in vivo studies by (Al-Hindi et al. 2019; Omodamiro et al. 2021) showed that high doses and prolonged use of the alcoholic extract of *G. latifolium* leaf extract may lead to hepatotoxicity.

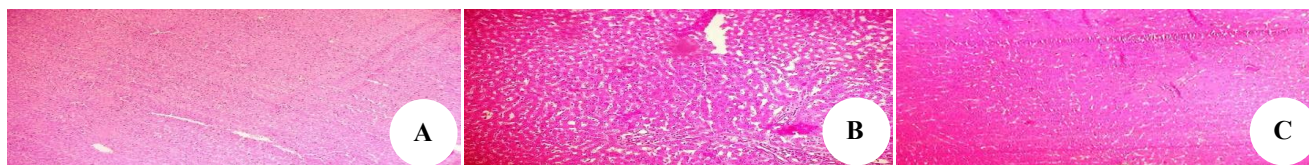


Figure 4. A. Plate 1: Photomicrograph of liver from group B (positive control), 10X (using Hematoxylin & Eosin staining e). It has normal hepatic tissue; B. Plate 2: Photomicrograph of liver from group C (negative control), 10X. It has normal hepatic tissue; C. Plate 3: Photomicrograph of liver from rat in group D (treated with 20% leaf extract and 80 % fruit extract, 10X) indicated normal liver tissue

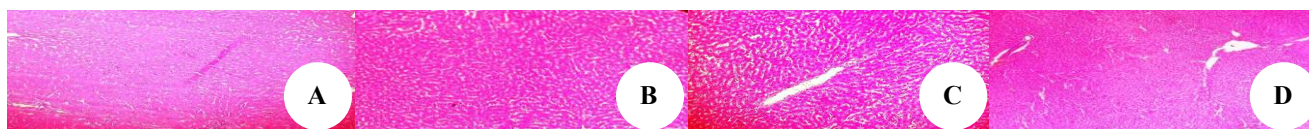


Figure 5. A. Plate 4: Photomicrograph of liver from the rat in group E (treated with 40% leaf extract and 60% fruit extract), 10X. It indicated normal liver tissue; B. Plate 5: Photomicrograph of liver from the rat in group F, 10X, treated with 50% leaf extract and 50% fruit extract, indicating normal liver tissue at 50 leaf:50 fruit extract of *Gongronema latifolium* NIF 2345; C. Plate 6: Photomicrograph of liver from rat in group G, 4X. It revealed normal liver tissue of rats treated with a 60:40 leaf:fruit ratio of 1 mg/kg extract; D. Plate 7: Photomicrograph of liver from the rat in group H (treated with 80% leaves: 20% fruit extract), 10X. It indicated normal hepatic tissue at 100mg/kg of leaf extract

The findings showed that groups D (20% leaf:80% fruit mg/kg), G (60% leaf:40% fruit mg/kg), and H (80% leaf:20% fruit mg/kg) show a decrease in serum creatinine and urea levels compared with the negative control, indicating that this plant may also possess hepato-renal protective function against naphthalene-induced damage (Ighodaro and Akinoye 2017; Ujong et al. 2022). Our findings align with recent studies (Okochi et al. 2024; Oyama and Chukwura 2024), suggesting that the protective effects of *G. latifolium* extract' on the liver and kidney are attributed to their phytochemical content, including alkaloids, tannins, flavonoids, and phenols, which exhibit antioxidant and anti-inflammatory properties.

The histology of the liver of all treatments showed normal hepatic tissue (Figures 4 and 5). The study showed that the extracts did not harm liver tissue in rats with naphthalene-induced oxidative stress, consistent with previous histopathological findings (Agwaramgbo et al. 2014). However, high fruit and leaf extract doses caused vacuolar degeneration, suggesting that moderate consumption is advisable.

In conclusion, the findings of this study highlight the significant hepato-renal protection and antioxidant properties of combined *G. latifolium* NIF 2345 extracts and reduced oxidative stress in naphthalene-induced rats. The study revealed that the LD₅₀ of the *G. latifolium* NIF 2345 extract was ≥ 5000 mg/mL BW, indicating that the extract is relatively safe, and the observed improvements in liver enzyme levels and body weight support its potential as a beneficial therapeutic agent in traditional medicine. The extract's administration reduced malondialdehyde levels and increased antioxidant enzyme activities. These findings suggest moderate consumption of the extracts, as they may offer a natural intervention against oxidative damage and liver toxicity. Further research is needed to explore the specific mechanisms behind the hepatoprotective and antioxidant activities of *G. latifolium* NIF 2345 extracts.

ACKNOWLEDGEMENTS

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Steroidal glycosides from *Mallotus philippensis* induce apoptosis in MCF-7 breast cancer cells via MTT and DAPI assays

HEMANTH KUMAR MANIKYAM^{1,*}, SUNIL K. JOSHI², SANDEEP BALVANT PATIL^{3,4},
SPANDANA VAKADI⁵, ABHINANDAN RAVSAHEB PATIL⁶

¹Department of Pharmacy, Faculty of Science, North East Frontier Technical University, National Highway 229, Aalo 791001, Arunachal Pradesh, India.
Tel.: +91-3783-222444, *email: drhemanthchem@gmail.com

²Department of Pediatrics, School of Medicine, University of Miami Miller, 1150 N.W. 14th St., Miami 33136, Florida, United States of America

³Dr. Shivajirao Kadam College of Pharmacy, Sangli 416305, Maharashtra, India

⁴Biocyte Institute of Research & Development, Sangli Bypass Road, Sangli 416416, Maharashtra, India

⁵Laughing Leaves Pvt Ltd, Hyderabad, Telangana 500018, India

⁶D Y Patil Education Society (Deemed to be University), Kolhapur 416006, Maharashtra, India

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Abstract. Manikyam HK, Joshi SK, Patil SB, Vakadi S, Patil AR. 2025. Steroidal glycosides from *Mallotus philippensis* induce apoptosis in MCF-7 breast cancer cells via MTT and DAPI assays. *Asian J Nat Prod Biochem* 23: 47-51. *Mallotus philippensis* (Lam.) Müll.Arg., commonly referred to as the kamala tree, is a widely known ethnomedicinal plant possessing diverse pharmacological activities. Despite its traditional applications, modern research into its anticancer properties remains underexplored. The present study investigates the in vitro anticancer activity of steroidal glycoside-enriched fractions derived from the aerial parts of *M. philippensis* against the MCF-7 breast cancer cell line. Ethanolic extraction followed by acetone precipitation was employed to obtain the glycoside-rich fraction. Phytochemical screening confirmed the presence of steroidal glycosides using Liebermann–Burchard and Salkowski tests. To evaluate cytotoxicity and apoptosis induction, MTT cell viability assay and DAPI nuclear staining assay were performed. The MTT assay results revealed a concentration-dependent inhibition of cell growth, achieving complete (100%) growth inhibition at 200 µg/mL. DAPI staining further confirmed the pro-apoptotic activity of the extract, showing prominent nuclear fragmentation and chromatin condensation, with an apoptotic index of $67.87 \pm 6.13\%$. These effects were comparable to the standard positive control, sodium Lauryl Sulfate (SLS), suggesting a potent anticancer potential. The findings support the hypothesis that steroidal glycosides present in *M. philippensis* can significantly induce apoptosis in hormone-dependent breast cancer cells. The anticancer effect is likely mediated through mitochondrial apoptotic pathways, characteristic of steroidal compounds. This study provides a strong rationale for further investigation into the bioactive constituents of *M. philippensis*, including isolation, molecular mechanism elucidation, and in vivo validation.

Keywords: Apoptosis, cytotoxicity, DAPI staining, *Mallotus philippensis*, MCF-7 cell line, MTT assay steroidal glycoside

INTRODUCTION

The kamala tree, which is also referred to as *Mallotus philippensis* (Lam.) Müll.Arg. is a medium-sized deciduous shrub of the family Euphorbiaceae. It is found in tropical and subtropical areas of the South and Southeast Asian regions and grows in dry and moist deciduous forests in countries such as India, Sri Lanka, Malaysia, and the Philippines (Ju et al. 2013; Tripathi et al. 2024). One of the distinguishing features of the species is its reddish-orange rind of the fruits is covered in a resinous exudate that possesses cultural, medicinal, and industrial significance. *Mallotus philippensis* has traditionally been a part of indigenous medicine that has been used for antiparasitic, hepatoprotective, anti-inflammatory, and antimicrobial purposes (Sumithira et al. 2022; Ali et al. 2024; Khadim et al. 2024). The red-colored powder on the fruit, a traditional natural dye and vermifuge, highlights the significance of the plant in traditional medicine and industry (Mao et al. 2019; Xiong and Long 2020). The medicinal potential of the plant is rooted in its rich phytochemical diversity. Secondary metabolites like polyphenols, flavonoids,

chalcones, tannins, and steroidal glycosides have been separated from different parts of the plant (Khadim et al. 2024). Of them, rottlerin is one of the most investigated constituents. The polyphenolic compound has various pharmacological actions, like anticancer, antioxidant, and anti-inflammatory activity. It acts through blocking protein kinase C-delta (PKC-δ), disrupting mitochondrial function, and triggering autophagy and apoptosis in cancer cells (Manhas et al. 2021). Other significant compounds are isorottlerin, kamala chalcones, and mallotophilippens and β-sitosterol-D-glucoside, which is responsible for the plant's synergistic therapeutic action (Pal et al. 2022). These compounds collectively enhance wound healing, inhibit microbial growth, and improve cytoprotection in human as well as veterinary use (Bodas et al. 2022; Wani et al. 2022).

The pharmacological actions of *M. philippensis* have a wide range. Its long-standing use as an anthelmintic, especially for the treatment of intestinal worms in children, is justified by the availability of chalcones and related constituents with established antiparasitic activity (Pal et al. 2022). Furthermore, fruit resin extracts exhibit considerable antibacterial and antifungal action, justifying their application

in skin infection treatment and wound healing. Flavonoids and polyphenols are responsible for antioxidant and anti-inflammatory effects by stabilizing free radicals and lowering oxidative stress, which is pivotal in the management of inflammatory disorders and cell damage (Sumithira et al. 2022). The anticancer activity of the plant is especially significant—rottlerin exhibited cytotoxicity, anti-proliferative, and pro-apoptotic activities against multiple cancer cell lines by targeting mitochondrial pathways and blocking PKC- δ (Lee et al. 2020; Sakthidhasan et al. 2022). Steroidal glycosides complement this action by mimicking the action of endogenous hormones and interfering with hormone-receptor pathways, which are highly applicable in hormone-sensitive cancers like breast cancer. Recent evidence highlights the prospect of steroidal glycosides as anticancer agents against hormone-dependent cancers, such as estrogen receptor-positive (ER+) breast cancer (Sakthidhasan et al. 2022). Their structural similarity to steroid hormones allows these compounds to regulate cell proliferation, apoptosis, and differentiation processes efficaciously (Manikyam et al. 2018).

Although steroidal glycoside-rich fractions from *M. philippensis* have shown promise in previous studies, their anticancer potential has not been thoroughly investigated. In this study, we evaluated the cytotoxic and pro-apoptotic effects of Iroid glycoside fractions on MCF-7 human breast cancer cells, a commonly used model for estrogen receptor-positive (ER+) breast cancer.

The research utilizes the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and DAPI (4',6-diamidino-2-phenylindole) assays for determining cell viability and apoptosis, respectively (Mosmann et al. 1983; Kumar et al. 2014; Balakrishna and Kumar 2015). The two-pronged approach facilitates the determination of the breast cancer lead molecule potential of *M. philippensis*-derived compounds. The research further seeks to complement ancient traditional herbalism with modern-day cancer pharmacology and thereby open up avenues for sustainable plant-derived therapeutics.

MATERIALS AND METHODS

Study area and sample collection

Aerial parts of *M. philippensis* were collected from the forests of Rajaji National Park, located in Haridwar, Uttarakhand, India (29.9373° N, 78.0891° E). Samples were shade-dried, powdered, and stored in airtight containers until extraction.

Preparation of herbal extract

Aerial parts of *M. philippensis* were used to extract the actives. The samples were air-dried in the shade, powdered, and treated with ethanol extraction (95%) under agitation at room temperature for 72 hours. The extract was filtered, concentrated by rotary evaporation, and precipitated with cold acetone (1:3 v/v) to eliminate polysaccharide and protein contaminants (Manikyam et al. 2017a,b). The pellet obtained was dried, weighed, and stored at -20°C prior to further analysis.

Preliminary phytochemical test for steroidal glycosides

Keller-Kiliani test (for cardiac glycosides)

To identify the occurrence of steroidal nucleus associated with sugar groups, typically present in cardiac/steroidal glycosides (Pal et al. 2022; Tripathi et al. 2024). Procedure: mix 1 mL of plant extract with 2 mL of glacial acetic acid containing a minute quantity of ferric chloride. Gently pour 1 mL of concentrated sulfuric acid down the sides of the test tube to create a distinct lower layer. Note the color change at the interface.

Liebermann-Burchard test (for phytosterols/steroids)

To identify unsaturated sterols and triterpenoids, particularly glycosylated ones like β -sitosterol-D-glucoside. (Pal et al. 2022; Tripathi et al. 2024). Procedure: Combine 2 mL of the ethanolic extract with 2 mL of chloroform in a dry test tube. Gradually add 2 mL of acetic anhydride and a few drops of concentrated sulfuric acid. Leave the mixture undisturbed for a few minutes.

Salkowski test (for steroids):

Add 2 mL of concentrated sulfuric acid to 2 mL of chloroformic plant extract slowly along the side of the test tube. Note the color at the interface (Pal et al. 2022; Tripathi et al. 2024).

MTT cell proliferation assay

The MCF-7 cell line was procured from the National Center for Cell Sciences, Pune, with a seeding density of 2.0×10^4 cells/well and was kept in liquid nitrogen for future testing. MTT (5 mg/mL) was dissolved in PBS and sterile filtered using a $0.22 \mu\text{m}$ filter, and it was utilized for the study as a stock solution (Manikyam et al. 2021).

Prior to assay, the test system MCF-7 cells were cultured at $37 \pm 1^{\circ}\text{C}$ in a gaseous atmosphere with $5\% \pm 1\%$ carbon dioxide in a humid environment in tissue culture flasks. The flasks contained 10% fetal bovine serum and Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) and penicillin (100 units) and streptomycin (100 μg) antibiotics (Invitrogen, USA) to have the subconfluence of cells (70 to 90% confluent); cell seeding for cytotoxicity testing.

The layers of the cells were washed with Phosphate Buffer Solution (PBS) and with 0.25% trypsin in 0.2 g/L EDTA in the culture flask at $37 \pm 1^{\circ}\text{C}$ until cells detached and floated, and DMEM with 10% FBS was then added to the flask to flush out the cells. Centrifugation was performed at 900 rpm for 5 minutes. DMEM was employed to suspend the cells, and cell counting was conducted to calculate the viability cell number/mL, and the cell number was adjusted to 2×10^5 cells/mL. Adjusted cells were taken at 0.1 mL and seeded in every well of 96-well plates. Mixing was done repetitively while seeding was performed to obtain a uniform cell suspension for plating the cells in every well. The well identified plates were incubated at $37 \pm 1^{\circ}\text{C}$ for 24 ± 1 h in gaseous atmosphere of $5\% \pm 1\%$ carbon dioxide and incubated for 24 ± 1 h. And cells were exposed to different concentrations of the test item. Spent medium was replaced with varying concentrations of test item solutions, and the incubation time to 48 ± 1 h at $37 \pm 1^{\circ}\text{C}$, in an atmosphere of $5 \pm 1\%$ carbon dioxide, with

positive, negative, and blank in assigned wells. Following incubation for 48 ± 1 h, the item and positive control medium were removed, and cells were incubated for 4 h with 20 μ L of MTT 5 mg/mL solution. Formazan crystals were developed in incubation by reduction of MTT and 150 μ L of DMSO was added to dissolve. The absorbance was measured at 570 nm after 10 min of incubation.

Reduction in the count of viable cells means a reduction in metabolic activity, resulting in a reduction in formazan formation. This direct relationship can be tracked by optical density at 570 nm. The formula for percentage viability is given below:

$$\% \text{ viability} = 100 (\text{O.D test item/O. D of control})$$

$$\% \text{ activity} = 100 - \% \text{viability}$$

DAPI nuclear staining assay

Cell seeding and treatment

MCF-7 cells were seeded onto sterile glass coverslips in 6-well plates at a concentration of 1×10^5 cells per well and incubated for 24 hours for cell adhesion. Cells were subsequently exposed to the IC₅₀ value of *M. philippensis* extract for 24 hours. Control cells were treated with DMSO alone (Manikyam et al. 2021).

Fixation and staining: Following treatment, cells were washed in Phosphate-Buffered Saline (PBS) twice and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and stained with 1 μ g/mL DAPI (4',6-diamidino-2-phenylindole) solution for 10 minutes in the dark.

$$\% \text{ of apoptotic cells} = (\text{apoptotic cells} + \text{late apoptotic cells}) / (\text{total number of cells}) \times 100$$

Microscopic analysis

The stained coverslips were mounted on glass slides using fluorescence mounting medium. Nuclear morphology was observed in a fluorescence microscope with an excitation wavelength of 358 nm and an emission wavelength of 461 nm. The apoptotic features of chromatin condensation and nuclear fragmentation were noted and compared with control cells (Manikyam et al. 2021).

RESULTS AND DISCUSSION

Keller-Kiliani test (for cardiac glycosides): A blue or green ring at the junction of the two layers, not confirmed, indicates the absence of a deoxy sugar moiety, indicating the presence of cardiac or steroidal glycosides. Liebermann–Burchard test (for phytosterols/steroids): The development of a blue-green color is a positive indication of the presence of steroidal or triterpenoid compounds, including their glycosidic forms. Salkowski test (for steroids): A reddish-brown ring at the interface is the indication of the presence of steroidal compounds.

MTT assay

Steroidal glycoside content was extracted from aerial parts of *M. philippensis* and screened by qualitative analysis, which was found to be positive for steroid and glycoside tests and negative for the rest of the tests as discussed. Pure steroidal glycoside was also characterized for its anticancer activity against MCF-7 cancer cell lines by DAPI and MTT assays. Steroidal glycoside exhibited >67% apoptosis activity against negative controls presented in Table 3. The result of the MTT assay indicated that the compound possesses the highest % average anticancer activity, 50%, at a 200 μ g/mL concentration in correlation with the positive test sample (SLS), as illustrated in Tables 1 and 2. *M. philippensis* extract-treated cells showed an IC₅₀ at a concentration (17.32 μ g/mL) when compared to SLS (107.02 μ g/mL) (Figure 2).

DAPI

The cells were seeded in a 24-well flat-bottom microplate with cover slips and kept at 37°C in a CO₂ incubator overnight. The 200 μ g/mL of compounds were treated at 48 hrs. Post incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. 20 μ L of DAPI was incubated for 5 min at room temperature in the dark, then observed under a fluorescent microscope. Randomly selected the fields in the microscope and counted the number of cells that underwent apoptosis, and calculated the percentage of apoptotic cells.

Morphological assessment of the nucleus by fluorescence microscopy with the help of cell-permeable nucleic acid stain, like DAPI, is normally employed for analysis of apoptosis. Steroidal glycoside extract of *M. philippensis* treated cells showed condensed chromatin with clear apoptosis when compared to the negative control using the DAPI assay method, as illustrated in Figure 1.

Table 1. % of apoptosis standard compound SLS

Concentration (μ g/mL)	% of Inhibition
25	22
75	45
100	58
200	88
400	100

Table 2. % of apoptosis test compound extract (*Mallotus philippensis*)

Concentration (μ g/mL)	% of Inhibition
25	66
50	88
100	95
200	100

Table 3. % of apoptosis test compound

Control	4.25 $\pm$ 2.90
Extract	67.87 $\pm$ 6.13

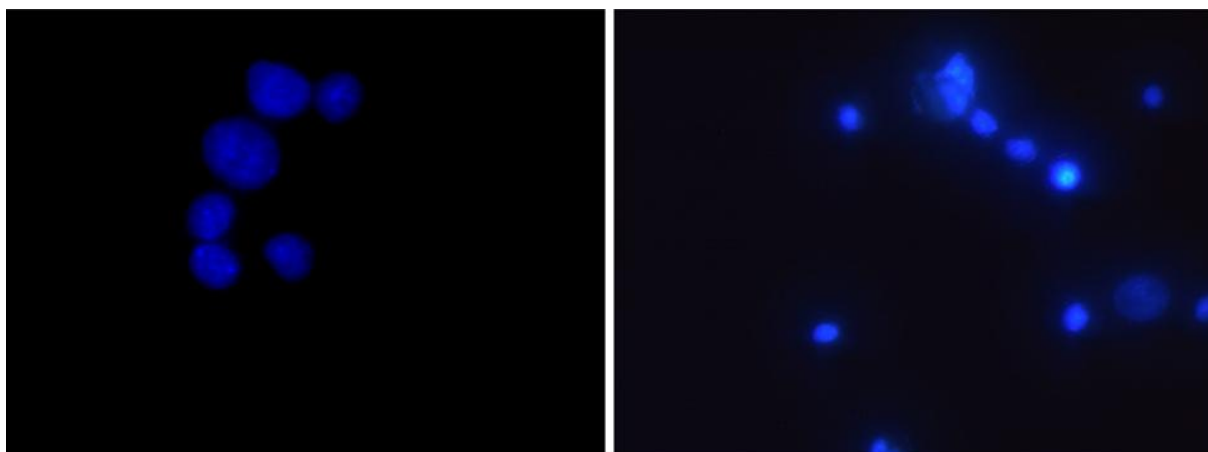


Figure 1. *Mallotus philippensis* extract-treated cells showed condensed chromatin, right image, with clear apoptosis when compared to the negative control, left image, using DAPI assay method



Figure 2. *Mallotus philippensis* extract-treated cells showed shown IC₅₀ at a lower concentration (17.32 µg/mL) when compared to SLS (107.02 µg/mL)

Discussion

The present study demonstrates that steroidal glycoside-enriched fractions derived from the aerial parts of *M. philippensis* exhibit significant anticancer activity against MCF-7 human breast cancer cells. The findings from both the MTT and DAPI assays confirm that these plant-derived compounds exert cytotoxic and pro-apoptotic effects at a level comparable to standard chemotherapeutic agents (Manikyam et al. 2021). Importantly, these results build upon and validate the ethnomedicinal use of *M. philippensis* in traditional systems for treating various ailments, including tumors and skin disorders. The MTT assay showed a concentration-dependent cytotoxic effect, with maximum inhibition of 100% observed at 200 µg/mL. This aligns with earlier reports where other phytosterol-rich or glycoside-containing plant extracts, such as those from *Psidium guajava* L. or *Paris polyphylla* Sm., have similarly shown

high cytotoxicity against hormone-sensitive cell lines like MCF-7. The ability of the extract to induce cell death at a relatively low concentration underlines its potential therapeutic potency and warrants further exploration of its constituent compounds.

Further validation was provided through DAPI nuclear staining, which revealed hallmark features of apoptosis such as nuclear condensation and fragmentation. The apoptosis index reached nearly 68% in treated cells compared to less than 5% in controls, indicating that the extract likely activates apoptotic pathways rather than causing nonspecific necrosis. This supports previous findings about the steroidal compounds like β -sitosterol-D-glucoside and rottlerin, which have been shown to induce apoptosis via mitochondrial pathways, caspase activation, and suppression of anti-apoptotic proteins like Bcl-2 (Manhas et al. 2021).

Interestingly, *M. philippensis* extract comparable activity with Sodium Lauryl Sulfate (SLS)—used as a positive control—demonstrates not just activity but efficacy. While SLS is a standard detergent compound known for causing cytotoxicity, its use in this study serves as a benchmark. The ability of a natural extract to perform on par with SLS emphasizes the potential of *M. philippensis* as a source of lead compounds for cancer alternative therapy.

It is also noteworthy that the plant is a rich source of multiple compound classes—roottlerin, iso-roottlerin, mallotophilippenes, kamala chalcones, and various triterpenoids—which may work in synergy. Synergistic interactions between different bioactive components can enhance efficacy and reduce toxicity, a strategy that has long been recognized in traditional polyherbal formulations (Manhas et al. 2021).

Moreover, these findings are relevant given the limitations of conventional chemotherapy, such as systemic toxicity, resistance development, and high cost. Natural products offer a sustainable, biocompatible alternative that could be harnessed either as primary therapeutics or as adjuvants to existing treatments. Particularly in low- and middle-income countries, affordable and accessible plant-based therapeutics can significantly improve cancer management outcomes. In vivo studies using xenograft models and toxicity profiling will also be crucial to establish clinical safety and efficacy. The pharmacokinetic properties of the lead compounds, including Absorption, Distribution, Metabolism, and Excretion (ADME) (Bodas et al. 2022), must be thoroughly investigated before advancing to preclinical or clinical trials.

This study provides the anticancer potential of *M. philippensis* steroidal glycosides and reinforces its value in modern pharmacognosy and drug discovery. The data support traditional claims and justify deeper pharmacological investigations, paving the way for novel anticancer agents from this underutilized medicinal plant.

This provides steroidal glycoside-enriched fractions of *M. philippensis* that have strong anticancer activity against the MCF-7 breast cancer cell line, causing profound apoptosis and growth inhibition similar to standard chemotherapeutic controls. *Mallotus philippensis* extract, especially enriched with lipophilic steroids and glycosides, is indicative of its ethnopharmacological significance and potential as a source of new drug development. Isolation of individual active compounds, determination of their molecular modes of action, and assessment of their efficacy in in vivo cancer models are suggested areas of further research.

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Review: The potential of freshwater eels (*Anguilla* spp.) as a source of medicine and food

DIO MEILANA ARIFIANDITA¹, FARIE AIMAR RAMADHAN¹, FENINTA SETYANING PARAMITA¹,
FITRIA PURNAMASARI¹, MUCHAMMAD SHOLIQIN¹, DARLINA MD. NAIM², AHMAD DWI SETYAWAN^{1,3,✉}

¹Department of Environmental Sciences, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57 126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ✉email: volatiloils@gmail.com

²School of Biological Sciences, Universiti Sains Malaysia. 1112, Persiaran Sains, 11800 Gelugor, Pulau Pinang, Malaysia

³Biodiversity Research Group, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia

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Abstract. Arifiandita DM, Ramadhan FA, Paramita FS, Purnamassari F, Sholiqin M, Naim DMd, Setyawan AD. 2025. Review: The potential of freshwater eels (*Anguilla* spp.) as a source of medicine and food. *Asian J Nat Prod Biochem* 23: 52-69. Freshwater eels (*Anguilla* spp.) possess exceptional nutritional and pharmacological properties that position them as a promising yet underutilized resource for functional foods and biomedicine. This review synthesizes current knowledge of their taxonomy, ecology, and life history traits, linking these to their biochemical composition and health benefits. Eel tissues are rich in high-quality proteins, long-chain omega-3 fatty acids (EPA and DHA), fat-soluble vitamins (A, D, E), and unique bioactive compounds such as glycosaminoglycans, peptides, and antimicrobial proteins. These constituents contribute to antioxidant, anti-inflammatory, immunomodulatory, anticoagulant, cardiovascular, and antimicrobial effects, with additional potential in neuroprotection, reproductive health, and skin repair. Comparative data across species and environments reveal substantial variability in nutrient profiles influenced by taxonomy, life stage, and habitat. The review also explores traditional and modern processing techniques that affect nutrient retention and bioavailability and discusses emerging applications in pharmaceuticals, nutraceuticals, and cosmetics. Recognizing the ecological threats to wild eel populations, the paper emphasizes the crucial role of sustainable utilization strategies, including aquaculture and value-added product development, in ensuring the conservation of this valuable resource. By integrating biological, nutritional, medicinal, and cultural perspectives, this review provides a comprehensive foundation for future research and innovation in eel-based health products.

Keywords: *Anguilla*, bioactive compounds, functional food, pharmacological properties, omega-3 fatty acids

INTRODUCTION

Wetlands are vital ecosystems that support biodiversity, regulate hydrological cycles, and provide numerous ecosystem services essential to both environmental sustainability and human livelihoods (Nygren and Lounela 2023). These areas, which include swamps, marshes, peatlands, and mangroves, play a key role in climate regulation, water purification, and the provision of habitat for aquatic and terrestrial species (Novita et al. 2022). Among the many resources offered by wetlands, freshwater eels (*Anguilla* spp.) stand out due to their ecological significance and economic potential.

Freshwater eels belong to the genus *Anguilla* within the family Anguillidae, and they are unique for their catadromous life cycle spawning occurs in the ocean. In contrast, most of their growth phase occurs in freshwater or estuarine environments (Arai 2020). This complex migratory behavior connects marine and freshwater ecosystems, highlighting their ecological importance. The genus comprises 19 species distributed across tropical, subtropical, and temperate regions (Froese and Pauly 2022), with species such as *Anguilla japonica* Temminck and Schlegel 1846, *Anguilla marmorata* Quoy and

Gaimard 1824, *Anguilla bicolor* McClelland 1844, *Anguilla rostrata* Lesueur 1817, and *Anguilla anguilla* Linnaeus 1758 showing varied adaptations to local environmental conditions (Arai and Abdul Kadir 2017).

Unfortunately, freshwater eel populations are experiencing dramatic declines due to a combination of overfishing, habitat destruction, pollution, and barriers to migration, such as dams and waterway modifications (Pike et al. 2020; Boubee et al. 2022). The European eel (*A. anguilla*), for instance, is currently listed as Critically Endangered by the IUCN Red List, while other species are also showing signs of stress (Moura et al. 2022). The decline in eel populations has raised significant concerns not only for ecological stability but also for communities that depend on eels for food, culture, and income (Chowdhury et al. 2020).

Despite increasing attention to their conservation status and aquaculture development, scientific studies on the medicinal and nutritional applications of freshwater eels remain limited and fragmented (Sila et al. 2018; Chowdhury et al. 2020). At the same time, eel ecology and biology are relatively well-documented (Arai 2016; Pike et al. 2020), but integrated reviews that bridge taxonomy, nutritional science, and pharmacological potential are still lacking (Santos et al. 2018; Lee et al. 2019). Given the global

momentum toward functional foods, natural therapeutics, and sustainable aquatic bioresources, a more comprehensive understanding of *Anguilla* spp. is urgently needed. This review aims to address these gaps and encourage further interdisciplinary research on their value as food and medicine (Rajasekaran et al. 2018; Rana et al. 2019).

In traditional medicine systems across Asia and parts of Europe, freshwater eels have long been used to treat ailments ranging from arthritis to skin conditions, reflecting a longstanding ethnomedicinal knowledge base (Kumar et al. 2017; Rajasekaran et al. 2018). In Japan, for instance, eel consumption is associated with health and vitality, particularly during the summer months. Meanwhile, in Indonesia, various ethnic communities use eel-based products as tonics or treatments for fatigue and joint pain (Wijayanti et al. 2018). However, scientific validation and consolidation of these traditional practices into structured biomedical frameworks remain limited.

With the growing global interest in functional foods and natural product-based pharmaceuticals, freshwater eels present a valuable yet underutilized resource. Their diverse species, wide distribution, and rich bioactive profile make them ideal candidates for further research and development in food and medicine. Nonetheless, unlocking their full potential requires an integrative understanding that encompasses taxonomy, physiology, ecology, nutritional composition, ethnomedicine, and sustainable harvesting and processing techniques.

This review aims to fill that gap by synthesizing available knowledge on the biological, nutritional, and pharmacological characteristics of freshwater eels. It highlights the interconnections between their life history traits and biochemical composition, evaluates their use in traditional and modern health applications, and explores opportunities and challenges for their sustainable utilization. Emphasis is placed on identifying species with the highest potential, understanding how environmental factors influence their nutrient content, and exploring innovative processing methods that preserve or enhance their medicinal properties.

Ultimately, this review aims to provide a scientific basis for future research and policy development on the responsible use of freshwater eels as a source of food and medicine. Hence, it contributes to the broader goals of food security, biodiversity conservation, and health promotion, particularly in regions where eel populations are integral to local ecosystems and cultures.

BIOLOGICAL CHARACTERISTICS RELEVANT TO NUTRITIONAL AND MEDICINAL USE

Taxonomy and distribution

The genus *Anguilla* comprises 19 recognized species and subspecies of catadromous freshwater eels that are globally distributed from tropical to temperate zones (Froese and Pauly 2022) (Table 1). These species are generally divided into tropical and temperate groups based on biogeographic range and habitat preference. Tropical species include *Anguilla marmorata* Quoy and Gaimard

1824, *Anguilla bicolor* subsp. *bicolor*, *Anguilla bicolor* subsp. *pacifica*, *Anguilla celebesensis* Kaup, 1856, and *Anguilla megastoma* Kaup 1856, predominantly found in Southeast Asia, Oceania, and parts of Africa. Temperate species, such as *Anguilla anguilla* Linnaeus 1758 (European eel), *Anguilla rostrata* Lesueur 1817 (American eel), and *Anguilla japonica* (Japanese eel), are common in Europe, North America, and East Asia (Tzeng et al. 2003; Arai 2016).

These eels share a unique catadromous life history: it is born in the sea, migrate to freshwater to grow, and return to the ocean to spawn. Despite similarities in the life cycle, *Anguilla* species have diversified significantly in morphology, genetics, and environmental tolerances, with distribution patterns strongly influenced by spawning sites, oceanic currents, and continental hydrology (Minegishi et al. 2005; Tsukamoto 2009). For example, *A. japonica* spawns in the western Pacific near the Mariana Ridge, and its larvae are transported via the Kuroshio Current to East Asian coasts (Tsukamoto et al. 2011). Similarly, *A. anguilla* originates in the Sargasso Sea and disperses across the North Atlantic to European inland waters (Pike et al. 2020).

From an evolutionary perspective, the Indo-Pacific region is hypothesized as the center of origin and radiation for the *Anguilla* genus (Arai and Chino 2012). This is supported by fossil records and molecular phylogenetic studies, which show a greater species richness and genetic divergence in tropical eels from this region (Minegishi et al. 2005). The existence of basal lineages such as *Anguilla borneensis* Popta 1924 and *Anguilla luzonensis* Watanabe, Aoyama and Tsukamoto 2009 reinforces the theory of tropical origin followed by dispersal to temperate zones through oceanic gyres and continental drift (Shiao et al. 2002).

The habitat range of *Anguilla* spp. is highly diverse. These eels are found in freshwater rivers, lakes, estuaries, brackish lagoons, and coastal wetlands. Some species, such as *A. marmorata* and *A. bicolor*, exhibit extraordinary ecological plasticity, capable of withstanding wide fluctuations in salinity, temperature, and oxygen levels. Studies in Indonesia and the Philippines have documented *A. marmorata* thriving in tidal swamps and mangrove fringes, environments characterized by high environmental stress and frequent salinity shifts (Arai and Abdul Kadir 2017). This adaptability has been linked to molecular and physiological traits, such as efficient osmoregulatory systems, respiratory plasticity, and antioxidant enzyme expression (Li et al. 2015; Wang et al. 2016).

Species distribution is not only a function of oceanic transport and habitat availability but is also influenced by competition, predation, and anthropogenic pressures. In regions with multiple co-occurring *Anguilla* species, niche partitioning is evident through habitat depth preferences, diel movement patterns, and dietary composition (Jellyman and Tsukamoto 2010). For instance, in the tropical rivers of Sulawesi and Sumatra, *A. celebesensis* tends to occupy upstream segments, while *A. marmorata* dominates lowland and estuarine zones (Kautsari et al. 2023). Such differentiation allows coexistence and may reflect underlying physiological specializations that affect not only habitat use but also tissue composition and nutrient accumulation.

Table 1. Overview of the 19 recognized species of genus *Anguilla*, their morphological traits, habitats, and distribution

Species name	Key morphological traits	Primary habitat	Geographic distribution	Reference
<i>A. anguilla</i>	Smaller head, narrow snout	Rivers, lakes	Europe, North Africa	Pike et al. (2020)
<i>A. australis australis</i>	Small eyes, blunt snout	Freshwater streams	Southeastern Australia, Tasmania	Arai (2022)
<i>A. australis schmidtii</i>	Variant of <i>A. australis</i> , minor color differences	Rivers	New Zealand	Arai (2022)
<i>A. bengalensis bengalensis</i>	Long dorsal fin, blunt snout	Rivers, swamps	Indian subcontinent	Kumar et al. (2017)
<i>A. bengalensis labiata</i>	Prominent lips, larger pectoral fins	Rivers	Sri Lanka, Southern India	Froese and Pauly (2022)
<i>A. bicolor bicolor</i>	Dark dorsal stripe, yellow belly	Rivers, lowland wetlands	Indian Ocean basin (South Asia, Indonesia)	Fahmi (2015)
<i>A. bicolor pacifica</i>	Lighter belly, elongated body	Coastal rivers, estuaries	Southeast Asia, Melanesia	Cabral et al. (2016)
<i>A. borneensis</i>	Fewer vertebrae, short dorsal fin	Blackwater rivers	Borneo	Fahmi (2015)
<i>A. celebesensis</i>	Short snout, blunt head	Highland rivers	Sulawesi, Java, Sumatra	Kautsari et al. (2023)
<i>A. dieffenbachii</i>	Large, robust body	Large rivers	New Zealand	Tesch (2003)
<i>A. interioris</i>	Small eyes, fewer vertebrae	Highland streams	Papua New Guinea	Froese and Pauly (2022)
<i>A. japonica</i>	Large eye, silvering during migration	Rivers, estuaries	East Asia (Japan, Korea, China)	Tsukamoto et al. (2011)
<i>A. luzonensis</i>	Distinct lateral line scales	Rivers	Luzon Island, Philippines	Minegishi et al. (2005)
<i>A. marmorata</i>	Marbled skin pattern, large size	Tropical rivers, brackish estuaries	Indo-Pacific, Indian Ocean, East Africa	Arai and Abdul Kadir (2017)
<i>A. megastoma</i>	Large mouth, deep body	Brackish lakes	Polynesia, Micronesia	Shiao et al. (2002)
<i>A. mossambica</i>	Uniform dark coloration	Coastal rivers	East Africa, Madagascar	Arai (2016)
<i>A. obscura</i>	Slender body, short fins	Estuaries, lowland rivers	Papua New Guinea, Fiji	Tesch (2003)
<i>A. reinhardtii</i>	Long body, distinct dorsal fin base	Rivers	Eastern Australia	Jellyman and Tsukamoto (2010)
<i>A. rostrata</i>	Slightly deeper body, pigmented leptocephalus	Coastal rivers, lakes	Eastern North America	Tesch (2003)

The nutritional and medicinal potential of freshwater eels is closely linked to their taxonomy and biogeography. Tropical eels such as *A. bicolor pacifica* and *A. marmorata* have been shown to exhibit higher fat reserves and distinct fatty acid profiles compared to temperate species, likely due to differences in water temperature, metabolic demands, and dietary availability (Ahn et al. 2015; Zhang et al. 2018). Moreover, regional variation in habitat types ranging from oligotrophic mountain streams to eutrophic wetlands can shape microbial exposure and immune responses in eels, potentially affecting the bioactive compounds found in their mucus and internal organs (Hilles et al. 2019; Chowdhury et al. 2020).

The decline in *Anguilla* populations, especially *A. anguilla*, has become a conservation concern globally. Overfishing, dam construction, and pollution have fragmented migration routes and reduced suitable habitats, leading to IUCN listings of several species as endangered or critically endangered (Pike et al. 2020). These pressures disproportionately affect temperate species with more defined and narrow migratory corridors. On the other hand, tropical species, due to their ecological flexibility and wider spawning seasons, may offer more resilient alternatives for aquaculture and resource utilization (Arai 2022).

Understanding species-specific distribution patterns and environmental requirements is essential for the sustainable

exploitation of eels for food and medicinal purposes. Identifying populations with high natural lipid and protein content, as well as those adapted to controlled environments, can guide selective breeding programs and targeted harvesting. Moreover, the characterization of the biochemical and genetic diversity within the genus *Anguilla* holds the potential for the discovery of novel compounds with therapeutic properties, such as glycosaminoglycans, peptides, and antioxidants, a prospect that is both intriguing and exciting (Sila et al. 2018; Bote et al. 2024).

In conclusion, the taxonomy and distribution of *Anguilla* spp. form the biological foundation for their significant roles in the application of food and medicinal industries. Their global distribution, physiological plasticity, and ecological versatility not only ensure wide availability but also contribute to their compositional richness. A comprehensive understanding of these aspects is, therefore, critical for effective conservation, aquaculture development, and bioresource utilization.

Morphology and physiology

The morphology of *Anguilla* spp. is characterized by a streamlined, elongated body that facilitates benthic locomotion and migration across varying aquatic environments. These eels typically exhibit a scaleless or minutely scaled integument that is enveloped in a thick

mucous layer. This unique and significant adaptation plays a vital role in protection against pathogens and assists in cutaneous respiration (Chowdhury et al. 2020). One of the most notable external features is the presence of a single continuous fin that merges the dorsal, caudal, and anal fins, allowing for sinuous, efficient swimming even in narrow crevices or low-flow aquatic systems (Tesch 2003). Pectoral fins are reduced in size, aiding their burrowing and substrate-associated behavior. The body coloration varies depending on species and developmental stage, ranging from transparent in the glass eel phase to dark green or yellowish-brown in adult yellow eels and eventually silvery in mature migrating eels (Righton et al. 2016).

Internal morphology reflects numerous physiological specializations. The musculature of *Anguilla* is highly developed and serves not only for locomotion but also as a major site of energy storage, particularly in the form of lipids during the yellow eel stage (Saito et al. 2015). Lipid content in muscle and liver increases significantly in preparation for the silver eel phase, which is marked by sexual maturation and long-distance migration to oceanic spawning grounds. These lipid reserves are critical, as the eels cease feeding once migration begins, relying solely on stored energy (Damsteegt et al. 2015).

Physiologically, freshwater eels are remarkably tolerant of environmental stressors such as salinity shifts, hypoxia, and temperature variation. A range of biochemical and molecular adaptations supports these tolerances. For instance, *A. marmorata* has been shown to upregulate antioxidant enzymes such as manganese superoxide dismutase (MnSOD) and copper-zinc superoxide dismutase (Cu/ZnSOD) in response to salinity changes, indicating a strong oxidative stress management system (Wang et al. 2016). Similarly, the expression of ion-regulating proteins like the vacuolar-type H^+ -ATPase (VHAB1) in the gills of *A. marmorata* increases under brackish and marine conditions, facilitating osmoregulation during habitat transitions (Li et al. 2015).

Another vital organ with physiological and nutritional significance is the corpuscle of Stannius (CS), a gland unique to fish and located near the kidney. In eels, the CS secretes stannioalcin, a hormone responsible for regulating calcium and phosphate levels in the blood, thus playing a role in bone formation and ionic homeostasis (Gu et al. 2015). This regulatory function is crucial not only for the eel's survival in different ionic environments but also suggests that tissues influenced by CS activity may possess mineral-rich biochemical profiles relevant to dietary health.

The swim bladder is another notable anatomical feature. In *Anguilla* spp., the swim bladder is physoclistous, meaning it is not connected to the digestive tract, and its gas content is regulated via the rete mirabile. This complex capillary system enables countercurrent gas exchange. This structure is supported by a gas gland that releases lactic acid and CO_2 to facilitate oxygen diffusion into the swim bladder lumen (Pelster et al. 2016). Importantly, the swim bladder tissue is rich in collagen and glycoproteins, compounds that have applications in nutraceuticals and cosmetics due to their role in skin elasticity and joint health (Caruso et al. 2020; Bote et al. 2024).

Hormonal control is also integral to eel physiology, particularly during the transition from yellow to silver eels. Endocrine changes include increases in gonadotropins and thyroid hormones that mediate gonadal development, metabolic regulation, and secondary sexual characteristics (Tzeng et al. 2003). These hormonal changes are also linked to shifts in muscle composition, where concentrations of Omega-3 Polyunsaturated Fatty Acids (PUFAs), especially Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA), increase significantly. These fatty acids are essential for human cardiovascular and neurological health and are a major reason why eel meat is valued as a functional food (Rana et al. 2019; Lee et al. 2019).

Additionally, the mucosal system of eels has garnered attention due to its antimicrobial and immunological properties. The mucus, secreted by goblet cells in the skin, contains a mixture of peptides, glycoproteins, and immunoglobulins that help protect against microbial invasion (Hilles et al. 2019). Some of these bioactive components have shown potential antibacterial effects against human pathogens, suggesting potential applications in the development of natural antibiotics or probiotic supplements (Rajasekaran et al. 2018).

Overall, the morphological and physiological features of *Anguilla* spp. underscore the species' ability to thrive in diverse and often harsh aquatic environments. More importantly, these characteristics directly contribute to the accumulation of bioactive compounds in their tissues, including omega-3 fatty acids, collagen, peptides, and essential minerals. These compounds not only support the survival and reproductive success of eels but also offer substantial nutritional and medicinal value for human consumption. Therefore, a deeper understanding of eel biology is essential to guide sustainable harvesting practices and optimize their utilization in health-related industries.

NUTRITIONAL COMPOSITION AND VARIABILITY

Macronutrients and micronutrients

Freshwater eels (*Anguilla* spp.) are considered nutritionally dense fish due to their high-quality protein and fat content. The protein content typically ranges between 15 to 20 g per 100 g of raw edible portion, depending on species and maturity stage (Ahn et al. 2015; Zhang et al. 2018). These proteins are easily digestible and possess a complete amino acid profile, including all essential amino acids required for human nutrition. The biological value of eel protein is considered comparable to that of eggs and milk, making it suitable for populations with limited access to conventional animal protein sources (Rana et al. 2019). Moreover, the relatively low collagen-to-muscle protein ratio enhances the meat's tenderness and palatability.

Fat content in eel meat is another notable attribute, with concentrations ranging from 5 to 15 g per 100 g depending on species, seasonality, diet, and physiological state (Saito et al. 2015). Tropical species such as *A. bicolor* and *A.*

marmorata have been reported to accumulate more intramuscular fat than temperate species, likely due to warmer aquatic environments and different energy storage patterns (Lee et al. 2019). This lipid fraction is particularly valuable as it contains a high proportion of long-chain omega-3 polyunsaturated fatty acids (PUFAs), notably eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids play well-established roles in cardiovascular protection, anti-inflammatory response, and brain development, contributing to the growing interest in eels as a functional food (Raatz et al. 2013; Calder 2015). The omega-3 to omega-6 ratio in eel fat is generally favorable, supporting anti-thrombotic and anti-atherogenic effects.

In addition to high-fat content, eel tissues also contain significant levels of phospholipids and sterols. Phospholipids contribute to membrane fluidity and cellular signaling, while cholesterol and related sterols serve as precursors for steroid hormones and bile acids (Caruso et al. 2020). In silver eels preparing for oceanic migration, lipid profiles shift to optimize energy availability and buoyancy control, which also influences the composition of edible tissues (Damsteegt et al. 2015). These dynamic changes in fat composition during life stages may impact the nutritional quality of harvested eels and should be considered in aquaculture or wild harvest strategies.

Eel meat is also rich in various essential micronutrients. Among minerals, phosphorus and calcium are present in relatively high concentrations, supporting bone development and energy metabolism (FAO 2021). Iron content in eel meat can reach up to 2.5 mg per 100 g, contributing to hemoglobin synthesis and oxygen transport (Lee et al. 2019). Other trace minerals such as magnesium, zinc, and selenium are also abundant, providing antioxidant protection, enzymatic cofactor functions, and immune support (Sila et al. 2018). Selenium, in particular, is notable for its synergistic role with vitamin E in preventing cellular oxidative damage.

The vitamin profile of freshwater eels includes lipophilic vitamins A, D, and E, along with several water-soluble B-complex vitamins. Vitamin A (retinol) is important for vision, epithelial maintenance, and immune competence, and its content in the eel liver is especially high, often exceeding that found in cod liver oil (Caruso et al. 2020). Vitamin D, primarily in the form of cholecalciferol (D₃), supports calcium absorption and bone health, making eels a valuable dietary source, particularly for populations at risk of deficiency. Vitamin E (tocopherol) functions as a major lipid-soluble antioxidant, helping to stabilize polyunsaturated fatty acids and prevent lipid peroxidation (Raatz et al. 2013). B-complex vitamins such as B₁₂, B₆, niacin, and riboflavin play crucial roles in energy metabolism and nervous system function.

Notably, the micronutrient composition of eels varies not only by species but also by environmental conditions and diet. Farmed eels fed formulated feeds enriched with fish oil and vitamin premixes tend to exhibit higher fat-soluble vitamin levels compared to wild-caught counterparts (Ahn et al. 2015). However, wild eels may accumulate more trace elements from natural diets, which

could offer additional health benefits or, conversely, pose risks due to bioaccumulation of contaminants such as mercury or Polychlorinated Biphenyls (PCBs) in polluted habitats (van den Heuvel et al. 2008). Therefore, understanding the source and condition of eels is crucial for maximizing nutritional benefits and minimizing potential hazards.

The high concentration of functional nutrients in eels makes them particularly suitable for vulnerable groups such as children, elderly populations, and individuals with high nutritional needs. Furthermore, the soft texture of eel meat and its savory flavor profile enhance its acceptability across cultures, supporting its traditional and modern culinary uses (FAO 2021). In many Asian countries, particularly Japan, China, and Indonesia, eel-based dishes such as *unagi kabayaki* or grilled *ikan sidat* are prized not only for taste but also for perceived health benefits, including stamina and recovery support (Tsukamoto 2009).

The increasing consumer demand for nutrient-dense and bioactive-rich foods offers opportunities to promote freshwater eels as a valuable component of healthy diets. However, sustainable sourcing, aquaculture optimization, and processing techniques must be refined to ensure the long-term viability of this resource. Comparative studies on species-specific nutrient profiles, seasonal variation, and the effects of cooking and preservation methods would further inform strategies to enhance eel-based food products. Further research on the nutritional composition of *Anguilla* spp. can help bridge the gap between traditional knowledge and evidence-based dietary recommendations.

Nutritional differences across species and environment

The nutritional profile of *Anguilla* spp. is not uniform across species or environments. Variability in fat, protein, mineral, and vitamin content has been documented based on differences in species, habitat conditions, feeding habits, sex, and life stage (Ahn et al. 2015; Zhang et al. 2018). For instance, *A. japonica* reared in intensive aquaculture systems tends to accumulate more intramuscular fat compared to wild-caught counterparts, largely due to energy-rich formulated feeds and restricted swimming space (Saito et al. 2015). Conversely, wild eels often display leaner muscle but may have higher micronutrient diversity derived from natural diets and varied aquatic environments (FAO 2021). These differences have implications for both food quality and health benefits derived from consumption.

Species-specific variation is especially evident in tropical vs. temperate eels. Tropical species such as *A. marmorata* and *A. bicolor* tend to exhibit higher lipid deposition and distinctive fatty acid profiles, which may be influenced by ambient water temperature and metabolic adaptation to warmer climates (Lee et al. 2019). *A. mossambica* from African rivers, for instance, exhibits lower protein-to-fat ratios than *A. anguilla* from colder European waters, possibly reflecting different energy storage strategies (Arai 2016). These patterns are important when selecting eel species for targeted nutritional formulations or functional food applications. Differences in

growth rate, metabolic rate, and diet type between species further amplify compositional diversity in eel meat.

Environmental conditions such as water salinity, temperature, and mineral composition also affect eel nutrient profiles. Studies on *A. bicolor pacifica* have shown that individuals from brackish environments possess higher ash and mineral content, especially calcium and magnesium, compared to those from freshwater rivers, likely due to differential ion availability in the water (Kautsari et al. 2023). Water quality factors, including pollution and dissolved oxygen, can also influence oxidative stress in eels, potentially altering antioxidant enzyme levels and associated nutrient markers (Wang et al. 2016). Thus, both natural and anthropogenic environmental variability must be considered when evaluating eel nutritional content.

Sexual dimorphism can further contribute to nutritional differences. Female eels often grow larger and store more lipids than males, particularly during the yellow and silver eel stages, when energy is reserved for reproductive migration (Righton et al. 2016). These differences are driven by the intricate hormonal regulation of metabolism and gonadal development, which not only affect total fat content but also influence the profile of bioactive lipids such as omega-3 fatty acids (Sila et al. 2018). In aquaculture settings, sex-related growth patterns may be manipulated through hormonal or environmental interventions to improve yield and nutritional output.

Life stage is another critical determinant of nutritional composition in *Anguilla* spp, during the transition from yellow eel to silver eel a process known as *silvering* profound physiological changes occur, including elevated fat accumulation, increased muscle mass, and enhanced antioxidant defenses (Tesch 2003). These changes prepare the eel for long-distance migration and reproductive success but also affect the quality of the meat harvested at this stage. Silver eels generally contain more DHA and EPA, along with higher levels of energy reserves, making them particularly valuable for both food and medicinal purposes (Ahn et al. 2015). However, post-spawning individuals exhibit depleted nutrient levels due to the energetic cost of migration and gametogenesis.

Regional variation in nutrition has also been noted across eel populations, even within the same species. For example, *A. bicolor* collected from different Indonesian rivers exhibits varying levels of zinc, selenium, and vitamin E, likely reflecting differences in local water chemistry and dietary sources (Kautsari et al. 2023). This intra-species variation poses challenges for the standardization of eel-based products, especially when used for health supplements or traditional medicine. Identifying and controlling for regional factors is essential for ensuring consistent product quality in commercial eel harvesting and processing.

Farming practices introduce another layer of variability. Eels raised under controlled aquaculture systems often receive high-protein diets supplemented with omega-3-rich fish oils and vitamin premixes, which may improve the nutritional quality of the meat (Rana et al. 2019). However,

confinement may limit physical activity and alter muscle structure, leading to differences in textural quality and fat distribution. On the other hand, semi-intensive or extensive systems that mimic natural environments may produce leaner eels with potentially richer micronutrient profiles but lower overall yield. Balancing these trade-offs is critical for sustainable and health-oriented eel production.

Understanding how species identity, life history stage, and environmental context influence the biochemical composition of eels is vital for optimizing their use in both food and pharmaceutical applications. These factors must be carefully accounted for in aquaculture protocols, wild harvesting practices, and regulatory frameworks governing food safety and nutritional labeling. Further research focusing on molecular and metabolomic profiling across species and habitats may reveal biomarkers associated with specific health benefits. This knowledge would greatly enhance the capacity to develop standardized, high-value eel-derived products for local and international markets.

PHARMACOLOGICAL AND MEDICINAL POTENTIAL

Anti-inflammatory and immune-modulating properties

Freshwater eels (*Anguilla* spp.) are emerging as a promising source of bioactive compounds with notable anti-inflammatory and immune-regulating effects (Table 2). Among these, protein hydrolysates and peptides generated through enzymatic processing have been shown to modulate inflammatory pathways by inhibiting the production of key pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) (Sila et al. 2018). These cytokines play central roles in the development of chronic inflammatory diseases, including arthritis, inflammatory bowel disease, and asthma. The ability of eel-derived peptides to downregulate these mediators suggests therapeutic potential for managing low-grade systemic inflammation and improving immune resilience.

In addition to cytokine modulation, eel peptides have demonstrated the capacity to inhibit pro-inflammatory enzymes such as Cyclooxygenase-2 (COX-2) and inducible Nitric Oxide Synthase (iNOS). These enzymes are responsible for generating inflammatory prostaglandins and nitric oxide, respectively, both of which contribute to pain, swelling, and tissue degradation (Park et al. 2017). Eel peptides can act at the transcriptional level, reducing gene expression of COX-2 and iNOS in activated immune cells such as macrophages and dendritic cells. This dual inhibition approach is valuable for attenuating both the initiation and amplification of inflammatory responses, thereby providing a broader anti-inflammatory effect compared to single-target agents. The potential of eel peptides to broaden the scope of anti-inflammatory effects is an intriguing area of research that could open up new possibilities for treating inflammatory conditions.

Table 2. Summary on *Anguilla* spp. (freshwater eels) chemical constituents, pharmacological properties, and medical utilization

Name	Chemical constituents	Pharmacological properties	Traditional medical utilization	Modern medical utilization	References
<i>A. anguilla</i>	GAGs, collagen, retinol, EPA, DHA	Anticoagulant, neuroprotective, anti-aging	Joint and nerve tonic	GAG-based anticoagulant, skin repair supplement	Arai (2016); Caruso et al. (2020); Bote et al. (2024)
<i>A. bicolor</i>	PUFA-rich oil, selenium, vitamin B complex	Anti-inflammatory, immunomodulatory	General health tonic	Peptide-based immune boosters, anti-inflammatory supplements	Ahn et al. (2015); Sila et al. (2018)
<i>A. bicolor pacifica</i>	PUFA, vitamins A & D, collagen	Antioxidant, skin repair	Used as a vitality enhancer	Functional food ingredient	Lee et al. (2019)
<i>A. celebesensis</i>	Calcium, vitamin D, peptides, collagen	Bone health, antibacterial	Postpartum recovery aid	Collagen supplements, antimicrobial peptides	Rajasekaran et al. (2018)
<i>A. japonica</i>	EPA, DHA, vitamin A & E, peptides, collagen	Cardiovascular, antioxidant, anti-inflammatory	Energy and stamina tonic	Functional foods, nutraceuticals, cosmetics	Sila et al. (2018); Lee et al. (2019)
<i>A. marmorata</i>	GAGs, peptides, vitamin D, minerals	Antimicrobial, anticoagulant, tissue repair	Wound treatment, vitality	GAG-extracts, topical antimicrobials	Chowdhury et al. (2020); Zhu et al. (2020)
<i>A. megastoma</i>	MUFAs, mucins, amino acids	Mucosal protection, fertility-enhancing	Aphrodisiac, energy enhancement	Fertility supplements	Arai (2016)
<i>A. mossambica</i>	Proteins, peptides	Antibacterial, antioxidant	Local medicine for infections	Topical wound-healing candidate	Arai (2016); Chowdhury et al. (2020)
<i>A. rostrata</i>	Omega-3 and -6, Zn, P, amino acids	Lipid-lowering, hepatoprotective	Liver tonic	Cardiovascular formulations	Calder (2015); Lee et al. (2019)

Note: The remaining species were categorized as *limited data*, *unknown*, or *unexplored* due to insufficient information.

Apart from peptides, eel tissues also contain immunomodulatory polysaccharides such as β -glucans and mannans, which are predominantly found in the mucus and connective tissues. These compounds stimulate immune responses in a balanced manner, enhancing host defense against pathogens while preventing hyperactivation that can lead to autoimmune disorders (Chowdhury et al. 2020). β -glucans are known to bind to pattern recognition receptors such as Dectin-1 and Toll-like receptors (TLRs), triggering signaling cascades that activate macrophages, natural killer cells, and neutrophils (Rajasekaran et al. 2018). Mannan, a polysaccharide composed of mannose units, may also support tissue regeneration and anti-inflammatory activity by modulating T-cell responses and promoting regulatory cytokine production.

The relevance of these bioactive components extends to clinical conditions characterized by chronic inflammation and immune dysregulation. For example, animal models of rheumatoid arthritis have shown significant reductions in joint swelling and cartilage erosion following oral or injectable administration of eel protein hydrolysates (Bote et al. 2024). Similar benefits have been observed in models of allergic airway inflammation, where eel-derived compounds reduced eosinophil infiltration, mucus hypersecretion, and airway hyperresponsiveness. These findings suggest potential applications of eel-based ingredients in managing asthma, atopic dermatitis, and other hypersensitivity-related disorders.

In the context of autoimmune diseases, where the immune system attacks its own tissues, modulation of T-cell activity is crucial. Eel peptides have demonstrated potential in promoting regulatory T-cell (Treg) populations while suppressing pro-inflammatory Th1 and Th17 cells, which are implicated in conditions such as multiple sclerosis, psoriasis, and inflammatory bowel disease (Sila et al. 2018). By shifting the immune balance toward tolerance and controlled inflammation, these compounds may offer a complementary approach to conventional immunosuppressants with fewer adverse effects. The capacity to induce a more regulated immune state is particularly relevant for chronic, relapsing conditions that require long-term management.

Bioavailability and delivery also play key roles in the effectiveness of these compounds. Studies indicate that low-molecular-weight peptides derived from eel muscle are more readily absorbed in the gastrointestinal tract, reaching systemic circulation and target tissues efficiently (Zhu et al. 2020). Additionally, encapsulation technologies such as liposomes and hydrogels are being explored to enhance stability and controlled release of eel bioactives in immune-modulating supplements. These delivery systems not only improve efficacy but also broaden the range of formulations for consumer use, including capsules, powders, and functional beverages.

It is also important to consider the synergy between different classes of eel-derived compounds. For instance, combining peptides with polysaccharides may offer additive or even synergistic effects on immune modulation, as each targets different components of the immune cascade. Peptides may primarily influence cytokine

production and enzyme activity, while polysaccharides engage innate immune receptors and modulate cellular activation. Formulating products that incorporate both components could result in broader-spectrum immune support suitable for a wide range of inflammatory and autoimmune disorders.

From a safety perspective, eel-derived compounds have generally demonstrated low toxicity and good tolerability in experimental studies. Unlike synthetic anti-inflammatory drugs, which often carry risks of gastrointestinal, renal, or cardiovascular side effects, bioactive peptides and polysaccharides from eels act through natural signaling mechanisms and are less likely to disrupt homeostatic functions (Calder 2015). Nevertheless, rigorous toxicological and allergenicity testing is essential before these compounds can be fully approved for use in pharmaceuticals or functional foods.

The potential of freshwater eels as a source of anti-inflammatory and immune-modulating agents represents a valuable convergence of traditional use and modern biomedical research. As chronic inflammation and immune-related disorders continue to rise globally, eel-derived bioactives may offer accessible, natural alternatives to synthetic therapeutics. Continued research, particularly clinical trials and mechanistic studies, will be key in validating these benefits and translating them into safe, effective, and market-ready health solutions.

Antioxidant activities

Freshwater eels (*Anguilla* spp.) are an excellent source of naturally occurring antioxidant compounds that protect biological tissues from oxidative damage. These compounds include Polyunsaturated Fatty Acids (PUFAs), particularly Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA), as well as vitamins A and E, and various endogenous peptides (Calder 2015; Lee et al. 2019). Antioxidants play a vital role in maintaining cellular homeostasis by neutralizing free radicals and Reactive Oxygen Species (ROS) that are implicated in aging, cancer development, cardiovascular disease, and neurodegenerative disorders (Raatz et al. 2013). Eel tissues, especially muscle and liver, are rich in these bioactives, contributing to their long-standing use in both food and traditional medicine to promote vitality and prevent disease.

The antioxidant capacity of eel-derived peptides is of particular interest due to their multifunctional roles. Protein hydrolysates produced through the enzymatic digestion of eel muscle yield low-molecular-weight peptides that exhibit strong radical scavenging activity in vitro, as measured by DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) assays (Sila et al. 2018). These peptides function by donating hydrogen atoms to unstable molecules, chelating metal ions, and inhibiting lipid peroxidation. Their amino acid composition particularly the presence of hydrophobic and aromatic residues like tyrosine, histidine, and methionine contributes to their high antioxidant potential (Zhu et al. 2020). Such peptides are being explored for use

in functional foods, dietary supplements, and even topical formulations targeting oxidative stress-related conditions.

Vitamin E (α -tocopherol), another key component of eel tissues, is a lipid-soluble antioxidant that protects cellular membranes from oxidative damage by scavenging lipid peroxyl radicals. It is particularly abundant in the muscle fat of tropical eel species such as *A. marmorata* and *A. bicolor pacifica*, which tend to accumulate more intramuscular lipids than temperate species (Ahn et al. 2015). In addition to preserving membrane integrity, vitamin E plays a role in gene expression regulation and modulation of immune responses. Similarly, vitamin A (retinol and its derivatives), found in high concentrations in eel liver, supports epithelial health and neutralizes singlet oxygen and peroxyl radicals, making it valuable for skin repair, immune modulation, and ocular health (Caruso et al. 2020). These vitamins, when consumed through eel products, contribute to the prevention of oxidative stress-associated diseases.

Lipid fractions extracted from eel tissues also display protective effects against oxidative stress in biological systems. EPA and DHA have been found to reduce oxidative markers and improve antioxidant enzyme activities, such as Superoxide Dismutase (SOD), catalase, and glutathione peroxidase, in animal models (Calder 2015). These omega-3 fatty acids are incorporated into cell membranes, where they modulate membrane fluidity and influence redox signaling pathways. Moreover, their metabolism leads to the formation of resolvins and protectins, lipid mediators that not only reduce inflammation but also enhance cellular defense mechanisms against oxidative injury. This dual action of PUFAs antioxidant and anti-inflammatory strengthens the therapeutic potential of eel oil in chronic disease prevention.

The commercial formulation *Eelax*, derived from eel extracts, exemplifies the application of these antioxidant principles in health product development. Although limited peer-reviewed studies are available on *Eelax*, preliminary reports and in vitro analyses suggest it reduces oxidative damage in human dermal fibroblasts and promotes collagen production, supporting claims related to anti-aging and skin repair (Bote et al. 2024). The efficacy is likely due to the synergistic interaction of eel peptides, collagen, and unsaturated fatty acids. Other formulations, especially in East Asian markets, incorporate eel-derived antioxidants into capsules, functional drinks, and cosmeceutical products aimed at combating fatigue, promoting skin elasticity, and supporting heart health.

Advances in processing technologies such as enzymatic hydrolysis and nanoencapsulation have further improved the stability and delivery of eel-based antioxidants. Nanoencapsulated eel oil has demonstrated improved resistance to oxidation during storage and better absorption in gastrointestinal models compared to non-encapsulated forms (Rajasekaran et al. 2018). These technologies are especially relevant for preserving the bioactivity of PUFAs and peptides, which are otherwise prone to degradation during conventional processing. The integration of such techniques into product development pipelines enhances

the feasibility of commercial antioxidant-rich eel products with extended shelf life and higher functional efficacy.

The bioavailability of eel-derived antioxidants is also a critical factor in determining their physiological effects. Studies have shown that peptides smaller than 3 kDa exhibit greater intestinal absorption and stronger antioxidant effects in cellular assays (Sila et al. 2018). Likewise, fat-soluble vitamins and omega-3s are better absorbed when consumed with dietary fats conditions naturally met in eel meat, which is rich in endogenous lipids. This intrinsic nutritional synergy allows for more efficient nutrient absorption and utilization, especially when eel is consumed as a whole food or minimally processed product.

Overall, the antioxidant properties of freshwater eels are supported by a diverse array of bioactive compounds that act through complementary mechanisms. Since oxidative stress is a common pathological feature in many chronic diseases, eel-derived antioxidants offer promising avenues for both preventive and therapeutic nutrition. Their continued study and development into validated products may contribute significantly to modern strategies in health maintenance, aging, and disease management.

Lipid regulation and cardiovascular health

Freshwater eels (*Anguilla* spp.) are an abundant natural source of long-chain Omega-3 Polyunsaturated Fatty Acids (PUFAs), particularly Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA), which have well-documented effects on cardiovascular health. These fatty acids are primarily found in eel muscle and liver, contributing significantly to their lipid profile and biological value (Lee et al. 2019). Unlike saturated fats, omega-3 PUFAs exert protective roles in the cardiovascular system by modulating lipid metabolism, reducing blood triglycerides, and improving overall cholesterol balance. Regular consumption of EPA and DHA is associated with lowered Low-Density Lipoprotein (LDL) cholesterol and elevated High-Density Lipoprotein (HDL) cholesterol, thus promoting a healthier lipid profile (Calder 2015). This balance is essential in reducing the risk of atherosclerosis and coronary artery disease.

EPA and DHA also influence blood pressure regulation through several interrelated mechanisms. These fatty acids enhance the production of Nitric Oxide (NO), a vasodilator that relaxes blood vessel walls and improves endothelial function (Raatz et al. 2013). Additionally, they reduce the synthesis of pro-inflammatory eicosanoids such as thromboxane A2 and leukotrienes, which are involved in vasoconstriction and platelet aggregation (Calder 2015). By reducing vascular inflammation and oxidative stress, eel-derived omega-3s improve vascular elasticity and lower systolic and diastolic blood pressure. Clinical and preclinical studies have confirmed that diets enriched with EPA and DHA can lead to modest but consistent reductions in blood pressure, particularly in individuals with hypertension (Kris-Etherton et al. 2002).

The anti-inflammatory properties of EPA and DHA are central to their cardiovascular benefits. Chronic low-grade inflammation plays a key role in the initiation and

progression of atherosclerosis, leading to plaque formation and hardening of the arteries. Omega-3 derived from eel modulates inflammatory signaling by downregulating nuclear factor-kappa B (NF- κ B) activity and reducing circulating levels of C-Reactive Protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- α) (Sila et al. 2018). This results in reduced leukocyte adhesion to endothelial cells and reduced macrophage infiltration into the arterial wall, thereby reducing the likelihood of plaque rupture and thrombosis. These effects highlight the potential role of eel fat in not only preventing but also attenuating the progression of existing cardiovascular disorders.

Beyond their systemic effects, EPA and DHA have been shown to alter lipid metabolism at the molecular level. They activate peroxisome proliferator-activated receptors (PPARs), particularly PPAR- α , which regulate the expression of genes involved in lipid oxidation and energy homeostasis (Calder 2015). This activation leads to increased fatty acid β -oxidation in the liver and reduced synthesis of triglycerides and Very Low-Density Lipoproteins (VLDL). These effects contribute to the lowering of circulating triglyceride levels, a key factor in metabolic syndrome and cardiovascular risk. In eel-fed animal models, reductions in hepatic fat accumulation and plasma triglycerides have been observed, providing experimental support for these lipid-lowering actions (Ahn et al. 2015).

The lipid composition of eel provides an additional advantage due to its favorable omega-3 to omega-6 ratio. Modern diets often contain excessive amounts of omega-6 fatty acids, which can trigger inflammatory pathways if not balanced with adequate omega-3 intake. Eel-derived oils offer a natural way to restore this balance, potentially reducing the risk of endothelial dysfunction, thrombosis, and arterial inflammation (Lee et al. 2019). This nutritional profile makes eel an appealing candidate for dietary intervention strategies aimed at improving cardiovascular health, particularly in aging populations or those at risk of metabolic disease.

Recent product development has begun incorporating eel oil into functional food formats and dietary supplements targeting heart health. In East Asian markets, soft-gel capsules containing purified eel oil enriched with EPA and DHA are marketed for supporting blood pressure regulation, cholesterol control, and cognitive function (Bote et al. 2024). Some formulations also include additional antioxidants such as vitamin E or coenzyme Q10 to enhance vascular protection further. Although consumer uptake is still modest compared to fish oil from anchovy or sardine, eel oil offers unique cultural, nutritional, and biochemical attributes that distinguish it within the omega-3 supplement space.

Processing and preservation methods significantly affect the stability and bioactivity of eel-derived lipids. Omega-3 fatty acids are prone to oxidation when exposed to heat, light, or oxygen, resulting in off-flavors and reduced efficacy. Technologies such as vacuum-sealing, refrigeration, and nanoencapsulation have been employed to enhance shelf life and preserve functional integrity

during storage (Rajasekaran et al. 2018). Flash freezing and microencapsulation of eel oil, in particular, have shown promising results in protecting PUFAs and ensuring consistent delivery of active compounds in supplement and food applications. These methods are vital for translating the cardiovascular benefits of eel-derived lipids into accessible, high-quality health products.

Eel-based sources of EPA and DHA offer an effective and culturally relevant means of addressing the growing burden of cardiovascular diseases worldwide. Their ability to modulate blood lipids, improve endothelial function, and reduce inflammatory responses positions them as valuable ingredients for dietary strategies and therapeutic interventions. Future clinical studies and long-term population data will be important in fully validating their role and optimizing formulation and dosage. As part of a balanced diet and integrated health approach, eel-derived lipids have the potential to have a significant impact on public health outcomes related to heart disease and metabolic dysfunction.

Anticoagulant effects

Glycosaminoglycans (GAGs), a class of linear polysaccharides found abundantly in animal connective tissues, have been identified in significant quantities within the skin and swim bladder of freshwater eels (*Anguilla* spp.), particularly the European eel (*A. anguilla*). These compounds, including dermatan sulfate and heparan sulfate, are structurally similar to pharmaceutical heparin and possess the ability to modulate blood coagulation pathways (Caruso et al. 2020). The anticoagulant action of eel-derived GAGs is mediated through their interaction with antithrombin III, a serine protease inhibitor that deactivates clotting factors such as thrombin and factor Xa. This mechanism prolongs clotting times and effectively reduces the risk of thrombosis, a major contributor to stroke, myocardial infarction, and venous thromboembolism (Zhu et al. 2020).

Unlike synthetic or mammalian-derived heparin, eel-based GAGs have demonstrated a promising safety profile. Studies have shown that these compounds inhibit coagulation without inducing hemolysis or triggering Heparin-Induced Thrombocytopenia (HIT), a serious side effect of conventional heparin therapy (Bote et al. 2024). This lack of hemolytic activity makes eel-derived anticoagulants an attractive alternative, especially for patients with heparin sensitivity or those requiring long-term anticoagulation. In vitro assays have confirmed that glycosaminoglycans from eel extracts can extend activated Partial Thromboplastin Time (aPTT) and Prothrombin Time (PT), both critical indicators of anticoagulant activity (Rajasekaran et al. 2018). These properties align with therapeutic goals in preventing clot formation without impairing platelet function or causing excessive bleeding, addressing the needs of heparin-sensitive patients, and fostering a sense of empathy and consideration in the audience.

The high concentration of GAGs in eel skin is associated with their role in maintaining tissue hydration, elasticity, and protection. These molecules are composed of

repeating disaccharide units, often sulfated, that carry a negative charge and bind to positively charged proteins and ions in blood plasma (Chowdhury et al. 2020). These interactions are key to their anticoagulant properties, as the sulfation pattern, particularly 2-O and 6-O sulfation, determines binding affinity to antithrombin and other regulatory proteins. The structural complexity of eel-derived GAGs may offer opportunities to fine-tune anticoagulant activity through purification and modification, potentially leading to more targeted therapies with reduced side effects.

Recent advances in enzymatic extraction and purification techniques have facilitated the isolation of bioactive GAGs from eel tissues in sufficient purity and yield for pharmaceutical evaluation. These methods typically involve protease digestion, ethanol precipitation, and ion-exchange chromatography to concentrate the anticoagulant fractions while removing contaminants and non-sulfated polysaccharides (Sila et al. 2018). Structural characterization using techniques such as Nuclear Magnetic Resonance (NMR) and mass spectrometry has confirmed the similarity of eel GAGs to low molecular weight heparins, though with distinct bioactive profiles. These methods also enable standardization, a key requirement for clinical development and regulatory approval.

Beyond their anticoagulant function, eel-derived GAGs may provide ancillary benefits in vascular protection and tissue healing. Their interaction with growth factors and adhesion molecules supports endothelial integrity and may reduce vascular inflammation (Calder 2015). These effects are particularly valuable in post-thrombotic care or as adjuvants to cardiovascular therapies. In animal models, administration of purified GAGs from eel extracts has led to reduced thrombus formation without impairing wound healing or triggering immune responses. However, it's important to note that there may be potential risks or limitations associated with their use, such as allergic reactions or immune system responses. These promising findings suggest broader applications for eel-derived compounds in integrative cardiovascular care, possibly in combination with other natural or synthetic agents.

It is important to note that the development of eel-based anticoagulants must consider ethical, ecological, and sustainability concerns. The European eel (*A. anguilla*) is classified as critically endangered due to overfishing and habitat loss (Pike et al. 2020). Therefore, sourcing GAGs from this species must be conducted responsibly, ideally from aquaculture or by utilizing byproducts of fish processing to minimize waste. Exploring alternative species with similar biochemical properties, such as *A. marmorata* or *A. bicolor*, may reduce pressure on vulnerable populations while expanding the resource base for drug development (Arai 2016). However, the real reassurance comes from the potential of eel aquaculture systems with controlled breeding and closed-loop production that could support sustainable access to raw materials for pharmaceutical applications.

From a regulatory perspective, further toxicological studies and human clinical trials are necessary before eel-derived GAGs can be approved as anticoagulant agents.

Although preliminary research supports their efficacy and safety, long-term data on pharmacokinetics, immunogenicity, and dose standardization are needed. Additionally, interactions with other anticoagulant drugs and possible contraindications must be assessed in diverse patient populations. Collaboration between biomedical researchers, pharmacologists, and regulatory agencies will be essential to guide the transition from laboratory research to clinical application.

Eel-derived glycosaminoglycans represent a compelling natural source of anticoagulant agents with distinct advantages over existing therapies. Their biochemical structure, multifunctional health effects, and relatively low toxicity profile position them as strong candidates for novel drug development. Continued innovations in extraction, purification, and formulation will likely accelerate their integration into pharmaceutical pipelines focused on cardiovascular and thrombotic disorders.

Antibacterial and antibiotic potential

Freshwater eels (*Anguilla* spp.) possess an innate defense system that includes a complex and biologically active mucus layer, which serves as a frontline barrier against microbial invasion. This mucus is rich in Antimicrobial Peptides (AMPs), lysozymes, proteases, and immunoglobulin-like proteins that collectively function to prevent bacterial colonization and infection (Chowdhury et al. 2020). These bioactive molecules are secreted in response to physical stress or environmental pathogens and are particularly concentrated along the skin and gill surfaces. AMPs from eel mucus typically exhibit cationic properties, allowing them to interact with negatively charged bacterial membranes, resulting in membrane disruption and cell death (Rajasekaran et al. 2018). Their spectrum of activity includes Gram-positive and Gram-negative bacteria, suggesting their potential as natural alternatives to synthetic antibiotics.

Several studies have confirmed the antibacterial efficacy of eel mucus extracts against clinically relevant pathogens. For instance, in vitro assays have shown that mucus-derived peptides from *A. japonica* and *A. marmorata* inhibit the growth of *Streptococcus mutans*, *Staphylococcus aureus*, and *Escherichia coli* (Bote et al. 2024). These effects are dose-dependent and occur at relatively low concentrations, supporting their use as biocompatible antimicrobial agents. In addition to killing planktonic bacteria, eel mucus peptides have been reported to disrupt bacterial biofilms, which are typically resistant to conventional antibiotics. This characteristic makes eel-derived AMPs particularly promising for applications in oral care products, wound healing formulations, and surface disinfectants.

The mechanism of action for eel AMPs involves both physical and biochemical pathways. Beyond membrane disruption, some peptides enter bacterial cells and interfere with DNA replication, protein synthesis, or metabolic enzyme function. Others act by chelating essential metal ions or modulating host immune responses to support bacterial clearance (Chowdhury et al. 2020). Unlike traditional antibiotics, which often target specific metabolic

pathways and are prone to resistance development, AMPs exert multi-targeted effects, reducing the risk of microbial adaptation. Their structural flexibility also allows for modifications that enhance stability, activity, or selectivity for specific pathogens.

In the context of aquaculture, particularly in Japanese eel farming (*A. japonica*), controlled use of antibiotics remains a subject of ongoing research. Pharmacokinetic studies of antibiotics such as amoxicillin in eels have revealed insights into absorption, distribution, metabolism, and excretion patterns unique to this species (Ahn et al. 2015). These studies help determine appropriate dosage regimes, withdrawal periods, and residue limits to ensure both therapeutic efficacy and consumer safety. However, overuse or misuse of antibiotics in aquaculture poses serious risks, including the development of antibiotic-resistant bacteria and contamination of aquatic ecosystems (FAO 2021). Therefore, integrating natural antimicrobial compounds such as eel AMPs into aquaculture management could reduce antibiotic reliance and support sustainable disease control.

The potential of eel mucus as a source of novel antibiotic agents is being explored in pharmaceutical research. Using modern extraction and purification techniques, such as ultrafiltration, reversed-phase chromatography, and mass spectrometry, researchers have been able to isolate and characterize individual peptides with potent antibacterial activity (Zhu et al. 2020). Some of these peptides share structural motifs with known antimicrobial families like defensins and cathelicidins, while others represent novel peptide classes. In experimental models, purified eel AMPs have demonstrated synergistic effects when combined with conventional antibiotics, lowering the Minimum Inhibitory Concentrations (MICs) required for pathogen inhibition and potentially mitigating resistance development.

Beyond antibacterial action, eel mucus peptides may exert immunomodulatory effects that enhance host defense mechanisms. By stimulating macrophage activation, cytokine secretion, and epithelial barrier function, these compounds contribute to a holistic antimicrobial defense system (Sila et al. 2018). This dual role of direct antimicrobial activity and immune enhancement positions eel mucus as a multifunctional bioresource with applications extending into immunotherapy, vaccine adjuvants, and antimicrobial coatings for medical devices. Moreover, topical formulations containing eel-derived peptides may be effective for treating skin infections or preventing microbial colonization in wounds and burns.

Despite their potential, challenges remain in developing eel-derived antibacterial agents for clinical and commercial use. Stability under physiological conditions, especially against protease degradation, is a key issue that must be addressed through peptide modification or encapsulation strategies. Additionally, large-scale production of AMPs from eel mucus is currently limited by low yields and extraction efficiency. Biotechnological approaches, such as recombinant peptide expression or synthetic peptide libraries, are being investigated to overcome these limitations and scale up production sustainably (Park et al.

2017). Regulatory pathways for the approval of AMP-based products must also be clarified, particularly regarding toxicity, allergenicity, and environmental impact.

The unique antimicrobial properties of eel mucus offer a compelling natural solution to rising concerns over antibiotic resistance and biofilm-related infections. With continued investment in extraction, characterization, and delivery technologies, eel-derived AMPs may soon play a role in the next generation of antimicrobial therapies. Their integration into aquaculture, food preservation, personal care, and clinical medicine reflects the broader applicability of natural peptides in addressing microbial threats across sectors.

Additional bioactivities

Beyond their widely studied antioxidant, anti-inflammatory, and cardiovascular properties, eel (*Anguilla* spp.) extracts exhibit a range of additional bioactivities with potential therapeutic applications. Among these are immunostimulatory, analgesic, reproductive, and neuroprotective effects, many of which are supported by the complex biochemical makeup of eel tissues. Eel-derived peptides, amino acids, and lipid fractions are thought to interact with physiological pathways that regulate immune function, pain perception, hormonal activity, and neural processes (Sila et al. 2018; Lee et al. 2019). Although research on these aspects is relatively nascent compared to mainstream pharmaceutical agents, the preliminary evidence offers strong justification for further exploration and development.

Immunostimulatory activity has been reported in various eel-derived compounds, particularly protein hydrolysates that enhance innate immune responses. Eel peptides have demonstrated the capacity to activate macrophages and natural killer (NK) cells, increase phagocytic activity, and stimulate the release of cytokines such as interleukin-10 (IL-10) and interferon-gamma (IFN- γ) (Zhu et al. 2020). These actions contribute to the enhancement of the host's non-specific immunity and may be beneficial in preventing infections, especially in immunocompromised individuals. Additionally, glycoproteins and mucins in eel mucus are believed to contribute to mucosal immune defense by promoting epithelial barrier function and limiting pathogen entry (Chowdhury et al. 2020). As interest grows in functional foods and natural immunomodulators, these compounds may serve as safe, food-derived alternatives to synthetic immune boosters.

Eel extracts have also shown potential in modulating pain and promoting analgesic effects. Although this property is less well-documented than others, studies using protein hydrolysates have reported reduced behavioral responses to noxious stimuli in animal models, suggesting antinociceptive activity (Rajasekaran et al. 2018). The mechanism is thought to involve modulation of inflammatory mediators and opioid-like pathways, although the exact peptides responsible remain to be fully characterized. Bioactive lipids, including DHA, may also contribute by altering membrane fluidity in neuronal cells and dampening neural transmission of pain signals (Calder

2015). This opens the possibility of developing eel-derived nutraceuticals or topical preparations for managing mild to moderate pain or inflammatory discomfort.

The reproductive health potential of eels is another area gaining scientific interest, particularly due to lipid and amino acid profile changes across their life cycle stages. During the silvering phase, when eels prepare for long-distance migration and reproduction, there is a notable shift in lipid metabolism and hormone levels, including increased accumulation of steroid precursors such as cholesterol and polyunsaturated fatty acids (Arai 2016; Righton et al. 2016). These compounds are essential for gonadal development and gamete maturation, and their concentrations suggest a biological alignment with reproductive function. Extracts from mature eel tissues may support fertility and hormone regulation, particularly in populations experiencing reproductive disorders associated with inflammation, oxidative stress, or hormonal imbalance. Some preliminary reports have even proposed eel oil as a supportive agent for testosterone balance and ovarian function, though human trials are lacking.

In addition to reproductive health, eel-derived compounds show promise in supporting neurological function. DHA, one of the dominant fatty acids in eel fat, plays a critical role in brain development, synaptic plasticity, and cognitive performance. It supports neuronal membrane integrity, modulates neurotransmitter activity, and exhibits neuroprotective effects by reducing neuroinflammation and oxidative damage (Raatz et al. 2013). Peptides with antioxidant and anti-inflammatory activities may also contribute to the prevention or delay of neurodegenerative conditions such as Alzheimer's and Parkinson's disease. Furthermore, the presence of bioactive amino acids such as tryptophan and tyrosine, which serve as precursors for serotonin and dopamine, indicates a potential role in mood regulation and stress response (Sila et al. 2018). These biochemical attributes align with growing interest in nutrition-based strategies for mental health support.

The multifunctional nature of eel bioactive compounds suggests that synergistic interactions between multiple compounds may enhance their overall efficacy. For instance, combinations of lipids, peptides, and trace elements such as zinc and selenium both present in significant quantities in eel tissues may collectively promote immune, endocrine, and neurological health (Lee et al. 2019). Such synergies could be harnessed in the design of integrated functional formulations that simultaneously target multiple physiological systems. This integrative potential aligns well with modern health paradigms that emphasize holistic, systems-level approaches to disease prevention and wellness maintenance.

While many of these additional bioactivities remain underexplored, they offer valuable directions for future research and innovation. Rigorous clinical trials and bioavailability studies are needed to validate the health effects of eel-derived compounds in human populations. Additionally, sustainable sourcing practices and standardized extraction protocols must be developed to

ensure consistent product quality and ethical use of eel resources. As scientific and consumer interest in multifunctional marine-derived bioactive compounds grows, freshwater eels may increasingly serve as the basis for next-generation functional foods and therapeutic agents targeting diverse health domains.

TRADITIONAL AND MODERN PROCESSING TECHNIQUES

Traditional practices

Traditional knowledge and culinary practices surrounding freshwater eels (*Anguilla* spp.) are deeply rooted in various cultures across Asia, Europe, and Africa. These practices reflect not only culinary preferences but also the need for preservation, given the perishable nature of eel meat and the seasonal availability of wild eels. Traditional methods emphasize flavor enhancement, texture modification, and microbiological safety without the use of modern refrigeration or synthetic additives (FAO 2021). Across cultures, eel preparation is often intertwined with ritual, identity, and medicinal beliefs, further elevating its value beyond mere sustenance.

In Indonesia, freshwater eels commonly referred to as *ikan sidat* are processed through several traditional techniques, including sun-drying, smoking, fermentation, and spice-rich cooking. One notable preparation is *rendang belut*, a regional variant of the Minangkabau dish *rendang*. Eel meat is simmered with coconut milk and a blend of spices until dry and darkened in color (Fahmi 2015). This method imparts antimicrobial properties due to the presence of turmeric, galangal, garlic, and chili, and also significantly extends the product's shelf life. In other parts of Indonesia, eels are sun-dried and salted, then fried or boiled before consumption practices that support rural preservation needs and facilitate transportation to urban markets (Arai 2016).

Japanese eel cuisine, particularly the dish *unagi kabayaki*, is among the most globally recognized eel preparations. The method involves filleting the eel, skewering it, and grilling it over charcoal while basting it with a sweet soy-based sauce made from mirin, soy sauce, and sugar (Tsukamoto 2009). This cooking process enhances umami flavor and produces a glossy caramelized exterior while maintaining the soft texture of the eel meat. Traditionally consumed during midsummer, *unagi* is believed to restore stamina and vitality, making it both a culinary delicacy and a seasonal health food (Lee et al. 2019). The cultural reverence for eel in Japan has also led to the development of refined culinary tools and preservation techniques, including vacuum-sealing and refrigeration for commercial *unagi* products.

In European countries such as the Netherlands, Germany, and the United Kingdom, eels are traditionally smoked or pickled. Smoking often involves hardwoods like oak or alder, which infuse the eel flesh with deep, earthy flavors while reducing moisture content and inhibiting microbial growth (Tesch 2003). Pickled eels are typically prepared in a vinegar brine infused with bay leaves,

mustard seeds, and black pepper, offering both preservation and palatability. In parts of Scandinavia, smoked eel is served as part of festive dishes, paired with rye bread or creamy sauces, reflecting its place in traditional gastronomy (Pike et al. 2020). These European practices often focus on enhancing flavor complexity and extending shelf stability, especially in coastal and riverine regions with strong fishing traditions.

In the Philippines, freshwater eels are commonly grilled, fried, or used in sour stews like *sinigang*, where tamarind-based broth helps tenderize the meat and neutralize its natural oiliness (Arai and Chino 2012). In some regions of Mindanao, eels are cooked in coconut milk with ginger and turmeric, reflecting the integration of local spices and indigenous techniques. Meanwhile, in Papua New Guinea and parts of Melanesia, *A. megastoma* and other native species are wrapped in banana leaves and baked in earthen ovens alongside root crops, illustrating low-tech but effective methods for flavor infusion and nutritional retention (Froese and Pauly 2022). These traditional methods are not only adapted to the local ecology but also often involve social cooperation and cultural transmission.

In African countries such as Madagascar and Mozambique, *A. mossambica* is caught using handmade traps and often grilled over open fires or stewed with local herbs. Smoking is also practiced in some fishing communities, especially in areas with poor access to refrigeration. Although less documented in scientific literature, these traditional practices are essential in sustaining rural protein sources and preserving biodiversity-related knowledge (Arai 2016). Moreover, the use of eel in folk medicine—as a tonic or restorative food remains embedded in oral traditions and community health beliefs.

Traditional processing methods can also affect the nutritional and medicinal properties of eels. Smoking and grilling may result in some loss of water-soluble vitamins, such as B-complex groups, but tend to preserve or even concentrate fat-soluble components like vitamin D and omega-3 fatty acids (Lee et al. 2019). Spice-rich preparations like *rendang* or *sinigang* may provide synergistic antioxidant and anti-inflammatory benefits through the combined action of eel lipids and botanical compounds (Sila et al. 2018). These practices, while developed in response to environmental and cultural contexts, align well with current functional food principles and require urgent further biochemical evaluation.

The continuity of traditional eel processing practices demonstrates a strong link between cultural heritage and food system resilience. These methods have evolved over generations to balance taste, preservation, and health, offering insights for modern product development and sustainable resource use. With growing interest in culinary biodiversity and ethnogastronomy, documenting and integrating traditional eel processing knowledge may contribute to food security strategies and smallholder economic development. The urgency of sustained interdisciplinary research is clear, as it can support

innovation that honors tradition while enhancing food safety and nutritional outcomes.

Modern innovations

Technological advancements in food science and biotechnology have opened new possibilities for processing and utilizing freshwater eels (*Anguilla* spp.) in more efficient, hygienic, and nutritionally optimized ways. Traditional methods, while valuable for cultural and practical reasons, often result in nutrient loss and limited shelf life. Modern innovations such as vacuum-sealing and flash freezing allow for the preservation of eel freshness and nutritional integrity during storage and distribution (FAO 2021). Vacuum-sealing helps prevent oxidative rancidity of omega-3 fatty acids, while flash freezing at ultra-low temperatures minimizes microbial growth and structural degradation. These technologies are essential for expanding eel export markets and ensuring safety in ready-to-eat products.

Ultrasound-assisted and microwave-assisted cooking methods have also been applied to improve the processing of eel meat. These techniques offer rapid and uniform heat transfer, reducing cooking time while preserving sensitive nutrients like vitamin A, vitamin E, and long-chain polyunsaturated fatty acids (Zhu et al. 2020). Additionally, ultrasound can enhance marination efficiency and meat tenderness by disrupting muscle fiber structure, making it useful in value-added eel products such as flavored fillets or jerky. Microwave cooking, on the other hand, enables energy-efficient heating and reduces the formation of undesirable compounds such as heterocyclic amines or polycyclic aromatic hydrocarbons compared to open-fire grilling (Lee et al. 2019). The integration of these methods can lead to healthier and more palatable eel-based food products.

A growing area of innovation involves the enzymatic hydrolysis of eel tissues to extract bioactive peptides. Controlled hydrolysis using enzymes such as pepsin, trypsin, or alcalase can release low-molecular-weight peptides with proven bioactivities, including antioxidant, anti-inflammatory, and antihypertensive effects (Park et al. 2017; Sila et al. 2018). These peptides can be incorporated into functional foods, beverages, or encapsulated supplements. Advanced purification methods such as ultrafiltration, ion-exchange chromatography, and reverse-phase HPLC are used to isolate specific peptides for research and product formulation. Optimization of hydrolysis conditions pH, temperature, time, and enzyme specificity—has become a key focus in maximizing bioactivity and yield.

In the cosmetics industry, eel skin and swim bladder have emerged as valuable sources of marine collagen. Collagen extracted from eel tissues shows high solubility, low viscosity, and favorable amino acid composition rich in glycine, proline, and hydroxyproline, all of which contribute to skin elasticity and repair (Caruso et al. 2020). Compared to bovine or porcine collagen, marine collagen from eel sources offers better absorption and lower allergenic potential, making it suitable for topical and oral applications. It is now used in a variety of cosmetic

products, including anti-aging creams, sheet masks, and collagen supplements, particularly in East Asian markets where marine-derived ingredients are culturally favored (Bote et al. 2024). The trend is further supported by consumer demand for clean-label, animal-free, and sustainable beauty solutions.

Another promising innovation is nanoencapsulation, which enables the delivery of bioactive compounds from eel extracts in targeted and protected formats. Nanoemulsions, liposomes, and biopolymer-based nanoparticles are being developed to improve the stability, solubility, and bioavailability of sensitive compounds like omega-3 fatty acids and peptides (Rajasekaran et al. 2018). This technology is particularly valuable in functional food and pharmaceutical sectors, where dosage control, taste masking, and prolonged release are crucial. Preliminary studies have shown that nanoencapsulated eel oil retains its bioactivity longer and is more efficiently absorbed in gastrointestinal models compared to non-encapsulated oil (Zhu et al. 2020). With further optimization, nanoencapsulation could enhance the efficacy of eel-derived products and facilitate their integration into complex formulations.

Modern packaging innovations also support the commercialization of eel-based products. Modified atmosphere packaging (MAP) and smart packaging systems with freshness indicators can help maintain product quality and inform consumers about storage conditions (FAO 2021). For instance, packaging eel fillets with nitrogen or carbon dioxide atmospheres reduces lipid oxidation and bacterial growth, thereby extending shelf life without chemical preservatives. In countries like Japan and South Korea, eel bento boxes with microwave-safe, vacuum-sealed compartments are now widely sold in convenience stores and online platforms, demonstrating the synergy of processing and packaging technologies.

Sustainability has also become a focus in modern eel processing innovations. With wild eel populations under threat, aquaculture systems have been developed to meet increasing demand without further depleting natural stocks. Intensive eel farming now utilizes Recirculating Aquaculture Systems (RAS) that control water quality, feeding, and growth conditions, resulting in standardized and traceable products (Arai 2022). These systems facilitate the production of high-quality raw material for modern processing while minimizing environmental impact. The combination of controlled aquaculture and precision processing strengthens the entire value chain for eel-based health and food products.

Collectively, these innovations demonstrate how traditional resources like freshwater eels can be elevated through science-driven approaches. The integration of advanced processing, extraction, and packaging technologies not only enhances product quality but also ensures safety, functionality, and consumer appeal. As research and development in this field continue to grow, freshwater eels are increasingly positioned as a valuable resource in modern food, health, and cosmetic industries.

Nutrient retention and bioavailability

Processing methods play a significant role in determining the nutritional quality and bioactivity of eel-based products. The thermal sensitivity of certain nutrients, particularly omega-3 Polyunsaturated Fatty Acids (PUFAs), antioxidants, and bioactive peptides, requires careful consideration during preparation. Among various cooking techniques, frying has been shown to cause the greatest loss of PUFAs due to oxidation and high-temperature degradation (Raatz et al. 2013). In contrast, gentler methods such as steaming and grilling at controlled temperatures tend to preserve omega-3 content and maintain antioxidant integrity (Zhu et al. 2020). These findings emphasize the importance of processing optimization for retaining the functional properties of eel nutrients.

Steaming and grilling also contribute to the preservation of water-soluble vitamins such as B-complex and vitamin C, which are otherwise prone to leaching and thermal destruction during boiling or deep-frying (FAO 2021). Studies comparing cooking methods have found that grilled eel retains higher levels of vitamin E and antioxidant enzymes compared to fried or microwaved samples (Lee et al. 2019). Furthermore, controlled grilling, as practiced in traditional Japanese *unagi kabayaki*, can result in a favorable flavor profile while minimizing nutrient loss. The Maillard reaction, which occurs during grilling, may also contribute to the formation of flavor-enhancing peptides and melanoidins with antioxidant properties (Sila et al. 2018). These combined effects reinforce the value of culturally informed yet nutritionally sound processing methods.

Marinating is another technique that has gained attention due to its dual role in enhancing and preserving flavor. The use of acidic or spiced marinades, such as those incorporating vinegar, citrus juice, turmeric, garlic, and chili, has been found to inhibit microbial growth while maintaining the integrity of key nutrients (Fahmi 2015). In eel processing, marinating not only improves palatability but can also enhance the shelf life without requiring synthetic preservatives. When paired with low-heat cooking methods or drying, marinating helps retain essential micronutrients like iron and zinc, as well as thermolabile compounds such as certain peptides and vitamins (Chowdhury et al. 2020). This makes marinated eel products suitable for both local consumption and value-added export markets.

Bioavailability, defined as the proportion of nutrients that are absorbed and utilized by the body, is influenced by both the intrinsic properties of eel tissues and the methods used for preparation. For instance, lipid-soluble nutrients such as vitamins A, D, and E show improved bioavailability when consumed with dietary fats, which are naturally present in eel meat (Calder 2015). However, excessive processing may denature protein structures or oxidize lipids, thereby reducing their digestibility and absorption efficiency (Park et al. 2017). Enzymatic hydrolysis, on the other hand, can improve peptide bioavailability by breaking down large proteins into absorbable fragments, a strategy often employed in

functional food and supplement development (Sila et al. 2018). This illustrates the potential synergy between processing technology and nutrient delivery.

Micronutrient retention also varies with storage and reheating practices. For example, repeated heating of eel dishes can degrade heat-sensitive compounds, including selenium-dependent enzymes and vitamin C. Packaging innovations such as vacuum-sealing and Modified Atmosphere Packaging (MAP) can help prevent oxidative degradation and preserve nutrient integrity during storage (FAO 2021). Additionally, the inclusion of natural antioxidants such as rosemary extract or tocopherols during processing has been shown to stabilize PUFA-rich oils in eel products, enhancing shelf stability and consumer safety (Caruso et al. 2020). These strategies collectively support the production of health-oriented eel products with extended usability.

Understanding the relationship between processing and nutrient dynamics is essential for product development, especially in the context of health claims and regulatory approval. For eel-based products marketed as functional foods, standardization of processing protocols is needed to ensure consistency in nutrient profiles and bioactive concentrations. This includes monitoring cooking time, temperature, pH, and the use of additives, all of which affect chemical composition and sensory properties (Lee et al. 2019). Digestibility and bioavailability studies using in vitro and in vivo models can provide further information for formulation strategies, ensuring that nutrients reach their intended physiological targets.

There is also growing interest in combining traditional and modern approaches to optimize nutrient retention. For example, integrating marination with vacuum-cooking (sous-vide) techniques can preserve thermosensitive nutrients while enhancing tenderness and flavor. Such hybrid methods draw on ethnogastronomic knowledge while applying scientific principles to control thermal exposure and oxygen contact (Zhu et al. 2020). These innovations are particularly relevant for high-value eel products aimed at premium health markets, where nutritional quality is a key differentiator.

Maintaining the functional value of eel nutrients requires an interdisciplinary understanding of food science, biochemistry, and culinary practice. As consumer interest in functional foods continues to grow, eel-based products must meet expectations not only for taste and convenience but also for scientifically validated health benefits. Advances in analytical methods, such as metabolomics and nutrient tracking, provide powerful tools to support this goal. Continued collaboration between researchers, processors, and traditional food practitioners can ensure that nutrient-rich eel products remain effective and culturally relevant across markets.

CHALLENGES AND FUTURE PERSPECTIVES

Despite the growing interest in the nutritional and medicinal potential of freshwater eels (*Anguilla* spp.), several challenges hinder their full utilization and

sustainable commercialization. One of the most pressing issues is the declining wild eel populations, particularly in temperate species such as *A. anguilla* and *A. japonica*, which are now listed as critically endangered and endangered, respectively, by the IUCN (Pike et al. 2020). Overfishing, habitat loss, pollution, and barriers to migration, such as dams, have severely disrupted their life cycles (Tsukamoto 2009). Conservation efforts are often complicated by the limited understanding of eel spawning behavior and migration routes in the open ocean, which makes population recovery strategies difficult to implement (Arai 2016).

Aquaculture offers a potential solution to reduce pressure on wild populations, yet it presents its own set of limitations. Eel farming is still largely reliant on wild-caught glass eels for seed stock, as artificial breeding in captivity has not yet been commercialized on a wide scale (FAO 2021). This dependence perpetuates the depletion of juvenile eel populations and creates bottlenecks in supply chains. In addition, intensive eel aquaculture requires high energy input, controlled water quality, and fishmeal-based feed, raising concerns about environmental sustainability and cost-effectiveness (Arai 2022). Research into captive breeding techniques, including hormonal induction and larval rearing, is progressing but remains technically and economically challenging.

Another significant challenge lies in standardizing the quality and safety of eel-based food and health products. Variability in species, habitat, diet, and life stage leads to inconsistent nutrient profiles, which complicates product formulation and quality control (Zhang et al. 2018). Moreover, eels are susceptible to bioaccumulation of environmental contaminants such as mercury, dioxins, and Polychlorinated Biphenyls (PCBs), especially in polluted waters (van den Heuvel et al. 2008). These substances pose health risks to consumers and complicate efforts to gain regulatory approval for eel-derived nutraceuticals and food ingredients. Ensuring traceability, implementing contaminant monitoring, and promoting clean aquaculture environments are necessary to mitigate these concerns.

From a processing standpoint, balancing nutrient retention with microbial safety remains a complex task. While modern technologies such as vacuum-sealing, enzymatic hydrolysis, and nanoencapsulation enhance product functionality and shelf life, their adoption requires investment in infrastructure, skilled personnel, and consumer education (Caruso et al. 2020) traditional methods. However, culturally significant, often lack standardization and fail to meet international food safety standards. Bridging the gap between traditional knowledge and modern technology is essential to ensure that eel-based products are both safe and accessible to a broad market.

Market development for eel-based functional products also faces cultural and perceptual barriers. In some regions, eels are perceived as exotic, oily, or unappetizing, limiting their acceptance among consumers unfamiliar with their culinary or medicinal value (Fahmi 2015). Furthermore, the premium price of eel products driven by limited supply, labor-intensive preparation, and processing costs may restrict access for lower-income populations. Marketing

strategies must therefore focus not only on health claims but also on taste appeal, cultural storytelling, and culinary versatility to broaden consumer acceptance.

In the scientific research field, strong clinical evidence is still needed to support the health benefits of eel-derived compounds. While *in vitro* and animal studies have shown promising antioxidant, anti-inflammatory, antimicrobial, and cardiovascular effects, human trials are still limited in scope and scale (Park et al. 2017; Sila et al. 2018). Regulatory agencies require comprehensive toxicological data, pharmacokinetics, and efficacy evaluations before allowing health claims on functional foods or supplements. Investment in interdisciplinary research that connects food science, pharmacology, and nutrition will be essential to translating preliminary findings into validated applications.

Moving forward, there are several opportunities to enhance the role of freshwater eels in nutrition, medicine, and sustainable food systems. Advances in biotechnology including molecular breeding, omics technologies, and synthetic biology could accelerate the domestication and scale-up of eel strains for aquaculture (Arai 2022). Precision aquaculture techniques using AI and sensor-based monitoring may improve the efficiency, welfare, and environmental performance of eel farms. Additionally, the development of plant- or microbial-based alternatives to fishmeal can improve the sustainability of eel diets without compromising their nutritional outcomes.

Policy support and international collaboration are also crucial for the long-term sustainability of eel resources. Harmonized regulations across countries on eel harvesting, trade, and aquaculture practices would improve transparency and conservation outcomes. Programs that integrate traditional ecological knowledge with scientific data, particularly in Southeast Asia, where eels play a role in local diets and medicine, could foster community-based management and benefit-sharing. Supporting smallholder farmer engagement and value-added processing may also contribute to rural development and food sovereignty.

With coordinated efforts in research, policy, industry, and community engagement, freshwater eels have the potential to become a sustainable and functional resource for future food and health innovation. Their unique combination of nutritional richness, pharmacological potential, and cultural heritage offers a compelling case for continued investment and responsible development.

CONCLUDING REMARKS

Freshwater eels (*Anguilla* spp.) have great potential as a multifunctional aquatic resource that bridges nutritional, medicinal, cultural, and economic domains. Their unique life history strategies, ecological plasticity, and biochemical richness position them as valuable sources of high-quality protein, essential micronutrients, and diverse bioactive compounds. Scientific research has increasingly validated the health benefits long associated with eels in traditional food and medicine systems, particularly their contributions to cardiovascular health, immune function, anti-inflammatory responses, and antioxidant defense. At

the same time, eel-based products are gaining commercial traction, with applications ranging from functional foods and dietary supplements to cosmeceuticals and therapeutic formulations. Technological innovations such as enzymatic hydrolysis, nanoencapsulation, and sustainable aquaculture systems are enabling the development of high-value, science-backed eel products with improved bioavailability and shelf life. However, challenges persist wild eel populations continue to decline due to overfishing, habitat fragmentation, and environmental contamination, raising conservation and traceability concerns. The variability in nutrient profiles across species, life stages, and environmental contexts further complicates standardization and regulatory approval of eel-derived products. Addressing these issues requires an integrated strategies that combine conservation science, modern aquaculture, rigorous safety monitoring, and ethical product development. Moreover, cultural knowledge related to traditional processing and therapeutic uses should not be overlooked, as it offers valuable insights into preparation methods that enhance nutrient retention and functional efficacy. Moving forward, interdisciplinary collaboration across food science, pharmacology, ecology, and policy will be essential to ensure the sustainable and equitable utilization of *Anguilla* spp. As global interest in natural and marine-derived health products continues to grow, freshwater eels offer a unique opportunity to contribute to public health, biodiversity conservation, and inclusive economic development, provided that innovation proceeds in tandem with sustainability and cultural respect.

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Review: Biological activity and zoochemical analysis of anchovies (*Stolephorus* spp.)

DIAN WIDOWATI¹, DAIVA A. NIRWASITA HARTONO¹,
FAUZAN RAFI WICAKSONO¹, EDRIC FARREL SAPUTRA¹, RAISA NOOR SAFIRA^{2,3}, CHEE KONG YAP⁴,
DARLINA MD. NAIM⁵, AHMAD DWI SETYAWAN^{1,6,✉}

¹Department of Environmental Science, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ✉email: volatileoils@gmail.com

²Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia

³Biodiversity Study Club, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia

⁴Department of Biology, Faculty of Science, Universiti Putra Malaysia. 43400 UPM Serdang, Selangor, Malaysia

⁵School of Biological Sciences, Universiti Sains Malaysia. 1112, Persiaran Sains, 11800 Gelugor, Pulau Pinang, Malaysia

⁶Biodiversity Research Group, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia

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Abstract. Widowati D, Saputra EF, Hartono DAN, Wicaksono FR, Safira RN, Yap CK, Naim DMd, Setyawan AD. 2025. Review: Biological activity and zoochemical analysis of anchovies (*Stolephorus* spp.). *Asian J Nat Prod Biochem* 23: 70-92. Anchovies (*Stolephorus* spp.) are small pelagic fish with broad distribution across tropical and subtropical marine ecosystems. They are ecologically significant as mid-trophic species and hold substantial value due to their rich nutritional and zoochemical composition. This review synthesizes current knowledge on anchovy-derived compounds, including high-quality proteins, essential amino acids, longchain omega-3 fatty acids (EPA and DHA), and bioavailable minerals such as calcium and selenium. These bioactive components exhibit diverse biological activities antioxidant, anti-inflammatory, antihypertensive, antidiabetic, and anticancer mediated through defined molecular mechanisms. Structure-function relationships and molecular interactions are examined in relation to their healthpromoting effects. The review also discusses technological innovations in extraction, processing, and formulation for functional foods and nutraceuticals. Environmental risks, including heavy metal bioaccumulation, microplastic exposure, and overfishing, are critically addressed along with strategies for sustainable harvesting and ecosystem-based management. Anchovies represent a promising marine bioresource for circular bioeconomy models, yet significant gaps remain in clinical validation, advanced compound characterization, and industrial-scale application. The multifunctional properties of anchovies support their integration into health-oriented and ecologically sustainable innovations, offering new opportunities in food, pharmaceutical, and environmental sectors.

Keywords: bioactive peptides, functional food, marine nutraceuticals, omega-3 fatty acids, *Stolephorus*, sustainable fisheries

INTRODUCTION

Anchovies (*Stolephorus* sp.) are small fish in the family Engraulidae. (Sosiawati 2019), widely distributed across tropical and subtropical marine ecosystems, including the Indo-Pacific region (Susanto et al. 2017; Sari et al. 2019). Characterized by their streamlined bodies, silvery coloration, and filter-feeding habits, anchovies play a vital role in marine food webs as both predator and prey (Checkley and Barth 2009; Pikitich et al. 2012). Their ecological importance is supported by their ability to regulate plankton populations while simultaneously serving as a food source for larger fish, marine mammals, and seabirds (Chaves and Umbria 2003; Tanaka et al. 2008). As such, anchovies contribute significantly to the sustainability and resilience of coastal marine ecosystems.

Beyond their ecological role, anchovies have long been recognized as a valuable component of traditional diets in many coastal communities. Rich in high-quality protein, essential fatty acids such as EPA and DHA, vitamins, and trace minerals like calcium, iron, and selenium, anchovies

represent a cost-effective source of animal-derived nutrition (Barros et al. 2014; Kari et al. 2022). These nutritional properties make anchovies not only important for addressing malnutrition and micronutrient deficiencies but also for supporting cardiovascular health, bone development, and immune function (Galasso et al. 2020a; Hu et al. 2020). The processing of anchovies into salted fish, fermented sauces, and dried snacks further enhances their accessibility and shelf life, especially in tropical developing nations.

Recent advances in marine nutraceutical research have highlighted the therapeutic potential of anchovies. Bioactive compounds derived from *Stolephorus* spp., including peptides, polyunsaturated fatty acids, and antioxidant enzymes, have shown promising pharmacological properties such as antihypertensive, antithrombotic, anti-inflammatory, antimicrobial, and antidiabetic effects (Mutalipassi et al. 2021; Shaik and Sarbon 2022b; Azfaralariff et al. 2023); these properties position anchovies as potential candidates for functional food development and novel marine-based health products.

In particular, fish-derived biopeptides have gained attention for their role in inhibiting free radicals and modulating key molecular pathways associated with chronic diseases (Hasari et al. 2021; Engwa et al. 2022).

While numerous studies have investigated the nutritional value of marine fishes in general, focused research on the zoochemical composition—defined as the spectrum of bioactive substances derived from animal tissues and biological activity of anchovies remains limited and fragmented. Zoochemical constituents in anchovies include not only macronutrients but also functional elements such as albumin, amino acids, marine enzymes, and trace hormones that may exert physiological effects in humans (Chen et al. 2022a; Nurmawati et al. 2022). A comprehensive synthesis of these components is needed to understand better their health implications, mechanisms of action, and potential applications in functional food and pharmaceutical development.

Moreover, global changes such as climate-driven shifts in marine productivity, increasing pollution from microplastics and heavy metals, and overexploitation of anchovy stocks pose challenges to the sustainability and safety of anchovy-derived products (Lima et al. 2022; Komala et al. 2024). However, understanding the interaction between anchovy biology, their zoochemical profiles, and environmental conditions is crucial for designing sustainable harvesting and processing strategies. Furthermore, evaluating the safety aspects, such as the potential accumulation of mercury or persistent organic pollutants, is essential for ensuring consumer health and regulatory compliance.

This review aims to bridge existing knowledge gaps by synthesizing current scientific literature on the biological activity and zoochemical analysis of anchovies (*Stolephorus* spp.). Specifically, it (i) explores the nutritional and bioactive profiles of anchovy tissues, (ii) summarizes known biological effects and underlying mechanisms of action, and (iii) evaluates environmental and health implications relevant to anchovy utilization. By integrating ecological, biochemical, and pharmacological perspectives, this review seeks to provide a holistic understanding of the role of anchovies in marine systems and human health.

This article also highlights the potential of anchovies as sustainable bioresources for future nutraceutical innovation, especially in the context of food security, marine conservation, and public health. The review concludes with directions for future research, including the need for standardized analytical methods, bioactivity-guided compound isolation, and toxicological assessments. It is hoped that this synthesis will serve as a valuable reference for marine biologists, food technologists, public health researchers, and policymakers working at the intersection of biodiversity conservation and human nutrition.

ECOLOGICAL AND TAXONOMIC OVERVIEW

Habitat and environmental adaptations

Anchovies (*Stolephorus* spp.) inhabit a range of tropical and subtropical marine environments, particularly warm and nutrient-rich coastal waters. These habitats are typically characterized by temperatures between 15-30°C and salinity levels close to 35 ppt, which are optimal for plankton production their primary food source (Pebruwanti and Fitriani 2018; Sari et al. 2019). Anchovies tend to dwell in the epipelagic zone up to 50 meters deep, where light penetration supports primary productivity and food availability.

They are commonly found in estuaries, coral reef fringes, bays, and river mouths, often aggregating in large schools to maximize feeding efficiency and predator avoidance (Prihantoko and Boesono 2019). Upwelling zones and coastal current systems are especially favorable due to the continuous supply of plankton and suspended nutrients. These physical conditions directly influence the distribution, growth, and reproductive success of anchovy populations.

A variety of physiological and morphological traits enables adaptation to these dynamic environments. Anchovies are capable of osmoregulation across varying salinity gradients, allowing them to survive in brackish and estuarine conditions (Falco et al. 2020). Their streamlined, hydrodynamic bodies facilitate fast and energy-efficient swimming. At the same time, their upward-facing mouths and elongated lower jaws enable rapid filtration of planktonic prey from the water column (Yusfiandayani et al. 2019). These morphological features are further complemented by reproductive strategies such as high fecundity and rapid larval development, allowing anchovy populations to recover quickly from environmental disturbances (Purnomo et al. 2017).

In addition to physical adaptations, anchovies exhibit behavioral and social traits that enhance survival. They often form synchronized schooling formations that provide hydrodynamic benefits and reduce individual predation risk through the confusion effect (Yoga 2019). Such schooling also facilitates efficient foraging in patchy plankton environments. Moreover, their migration patterns are influenced by seasonal temperature shifts and plankton blooms, ensuring continued access to food and suitable spawning grounds.

The ecological flexibility of anchovies is further demonstrated by their presence in various microhabitats and fluctuating coastal systems. Their abundance in highproductivity areas makes them a key trophic link in marine food webs, transferring energy from primary producers (phytoplankton and zooplankton) to higher-level predators, including commercial fish species, marine mammals, and seabirds (Pikitch et al. 2012). Consequently, anchovies are considered important ecological indicators of coastal ecosystem health. To support this description, spatial data on the environmental preferences of anchovies can be illustrated in Figure 1 and Table 1.



Figure 1. Typical habitats of *Stolephorus* spp. in tropical coastal ecosystems

These environmental parameters define anchovy habitat selection and underpin their seasonal migration, spawning success, and abundance across Indo-Pacific waters. Understanding these ecological preferences is crucial for effective management of anchovy fisheries, particularly in the face of climate-driven oceanographic changes and coastal habitat degradation (Costalago et al. 2011).

Species distribution and invasiveness

Anchovies (*Stolephorus* spp.) are widely distributed throughout tropical and subtropical waters, especially in the Indo-Pacific region, extending westward from the Indian Ocean to the western Pacific Ocean. Their primary habitats include coastal zones of Southeast Asia, northern Australia, the Bay of Bengal, and surrounding archipelagic waters (Cavraro et al. 2022; FishBase 2024). Their physiological tolerance to variable salinities and temperatures, as well as

their plankton-based diet, which is abundant in many coastal ecosystems, facilitates this broad distribution.

In Indonesia, anchovies are commonly found in the Java Sea, Sulawesi Sea, and waters surrounding the Lesser Sunda Islands (Andriyono 2018; Yonvitner et al. 2020). Their occurrence in shallow marine areas, estuaries, and reef-associated systems reflects their ecological plasticity. Seasonal spawning migrations enable them to exploit nutrient-rich areas, particularly during monsoonal-driven upwelling events. During such periods, anchovy populations can increase dramatically, forming large shoals that are both ecologically and commercially significant (Masyhudi 2024).

The dispersal potential of *Stolephorus* spp. also raises concern regarding their invasive tendencies in non-native environments. While not traditionally considered invasive, their high fecundity, broad salinity tolerance, and rapid growth rates make them capable of establishing populations in new areas if accidentally introduced (Lima et al. 2022). In some regions, such as the Caribbean and Gulf of Mexico, closely related anchovy species have been observed exhibiting non-native spread, potentially competing with local pelagic fish for food resources.

A major ecological concern is their ability to outcompete native plankton-feeding species due to their efficient foraging and reproductive capacities. In ecosystems where trophic balance is fragile, such introductions could result in shifts in plankton community structure, reduction in native species abundance, and alterations in nutrient cycling (Smith and Jones 2023). Moreover, anchovies may serve as vectors for pathogens or parasites if transported via ballast water or aquaculture effluents.

Despite their broad range, most *Stolephorus* species remain underrepresented in global conservation databases. According to the IUCN, many anchovy species are listed as Least Concern (LC) or Data Deficient (DD) due to limited population data and taxonomic resolution (Fricke et al. 2019; GBIF 2024). This underscores the need for increased taxonomic and ecological research, especially in regions where their populations are heavily exploited or where potential invasive pathways exist. To visualize their distribution and assess invasiveness risk, see Figure 2 and Table 2.

Table 1. Summary of habitat types and environmental adaptations of *Stolephorus* spp.

Habitat type	Environmental characteristics	Anchovy adaptations
Coastal pelagic zones	Warm, shallow seas (20-30 °C), high light penetration	Streamlined body for fast swimming; filter-feeding on plankton
Estuaries and river mouths	Variable salinity (brackish), high nutrient input	Osmoregulatory capability; tolerance to salinity gradients
Coral reef fringes	Clear waters, structural complexity	Schooling behavior for predator avoidance: high maneuverability
Upwelling zones	Cool, nutrient-rich currents, high plankton concentration	High reproductive capacity; rapid growth linked to food abundance
Bays and lagoons	Sheltered, productive inshore environments	Opportunistic feeding; aggregation in large shoals for foraging efficiency
Epipelagic layer (0-50 m)	Oxygen-rich, photic zone with abundant zooplankton	Upward-facing mouth and elongated jaw for plankton capture

Sources: Prihantoko and Boesono (2019); Sari et al. (2019); Yusfiandayani et al. (2019); Falco et al. (2020)

Table 2. Major *Stolephorus* species and their distribution across ocean regions

Species	Native range	Reported non-native occurrence	Invasiveness risk	IUCN Status
<i>S. indicus</i>	Indian Ocean, Southeast Asia	Red Sea (occasional)	Low-Moderate	Least Concern
<i>S. commersonnii</i>	Western Pacific, Indonesia, Australia	Caribbean Sea (introduced records)	Moderate	Data Deficient
<i>S. dubiosus</i>	Indo-Malay waters	No records	Low	Not Evaluated
<i>S. waitei</i>	Papua New Guinea, Northern Australia	No records	Low	Least Concern
<i>S. insularis</i>	Western Indian Ocean	Not documented	Unknown	Not Evaluated

Sources: Fricke et al. (2019); Lima et al. (2022); FishBase (2024); GBIF (2024);

**Figure 2.** Global distribution map of anchovy (*Stolephorus* spp.) species across Indo-Pacific regions

Understanding both the natural distribution and potential for ecological spread is critical for managing anchovy populations (Abdusysyahid 2024). In regions where fisheries are expanding or shipping intensity is high, monitoring programs are essential to detect possible introductions and assess ecological impacts. Moreover, accurate species identification is vital, as cryptic diversity within *Stolephorus* may obscure invasion dynamics and conservation priorities.

Taxonomic classification and morphology

Anchovies of the genus *Stolephorus* are members of the family Engraulidae, order Clupeiformes, class Actinopterygii, phylum Chordata, and kingdom Animalia (GBIF 2024). This taxonomic grouping places them among ray-finned fishes with a close evolutionary relationship to herrings and sardines. The genus *Stolephorus*, first described by Lacépède in 1803, comprises numerous species that are morphologically similar but differ in subtle anatomical features, meristic counts, and geographical distributions (Hata and Motomura 2022). Most species are small, typically ranging from 5 to 15 cm in length, and are easily recognized by their slender bodies, prominent silver lateral bands, and terminal or slightly upturned mouths.

Morphologically, *Stolephorus* spp. exhibit adaptations that support their pelagic lifestyle and plankton-feeding behavior. Their bodies are laterally compressed and covered in thin cycloid scales that reflect light, aiding in

camouflage in the epipelagic zone. The head is moderately large with a terminal to slightly superior mouth, suited for filter feeding on plankton. Notably, the lower jaw tends to protrude beyond the upper jaw, which enhances surface skimming efficiency during foraging. The dorsal fin is short and positioned near the middle of the body, while the anal fin is relatively longer, contributing to maneuverability. The caudal fin is deeply forked, enabling fast swimming and sudden directional changes, an important trait for evading predators and maintaining schooling formation (Yusfiandayani et al. 2019).

Internally, anchovies possess a well-developed gill raker system used to trap fine planktonic particles. The vertebral column is flexible, providing agility in water, and the swim bladder is reduced or absent in many species, reflecting their active, near-surface lifestyle. Sexual dimorphism is minimal, although during the spawning season, females may exhibit slightly enlarged abdomens. Eggs are pelagic and spherical, typically hatching within 24–48 hours after external fertilization in warm, plankton-rich waters (Gopinath and Abdussamad 2024).

These morphological and anatomical traits not only support the ecological niche of anchovies but also serve as taxonomic markers in species identification. Due to the subtle differences between species, accurate identification often requires detailed examination of gill raker counts, scale patterns, and the shape of the supramaxilla bones. Advances in molecular genetics and morphometric analysis are increasingly used to resolve taxonomic ambiguities within the genus. Such precision is particularly important in ecological studies, fisheries management, and when evaluating the bioactivity or biochemical properties of specific *Stolephorus* species (Table 3; Figure 3).

Ecological roles and conservation status

Anchovies (*Stolephorus* spp.) occupy a pivotal ecological niche in tropical and subtropical marine ecosystems as midtrophic-level organisms. Functioning both as consumers of plankton and as prey for higher-level predators, they serve as a crucial energy conduit linking primary producers with piscivorous fish, marine mammals, and seabirds (Tanaka et al. 2008; Pikitich et al. 2012). By intensively consuming phytoplankton and zooplankton, anchovies help regulate planktonic community dynamics and influence nutrient cycling through the excretion of nitrogen and phosphorus compounds (Checkley and Barth 2009). Their dense schooling behavior also plays a role in predator-prey interactions by shaping the foraging strategies of larger pelagic species such as tuna, mackerel, and dolphins (Figure 4).

Table 3. Morphological characteristics used in taxonomic identification of *Stolephorus* spp.

Morphological trait	Diagnostic relevance	Variation among species
Body shape and size	General body form (slender, compressed), total length	Typically 5-15 cm; some species reach >18 cm
Lateral silver band	Presence, width, and reflectivity of lateral stripe	Prominent in most species; varies in length and intensity
Mouth orientation	Terminal or slightly superior mouth; jaw projection	Lower jaw often protrudes beyond upper jaw
Gill raker counts	Number and density of gill rakers on the first branchial arch	Used to differentiate plankton-feeding adaptations
Scale type and arrangement	Type (cycloid), scale size, and pattern along the lateral line	Species-specific scale patterns and size
Supramaxilla bone shape	Subtle anatomical differences in the upper jaw bone structure	Key for species-level separation
Fin position and shape	Dorsal, anal, and caudal fin characteristics	Dorsal fin mid-body; anal fin relatively long
Swim bladder presence	Reduced or absent in many species; aids in pelagic adaptation	Absence supports near-surface swimming behavior
Egg morphology (when known)	Shape and buoyancy of pelagic eggs (for reproductive identification)	Spherical, transparent, planktonic

Sources: Yusfiandayani et al. (2019); Hata and Motomura (2022); Gopinath and Abdussamad (2024)

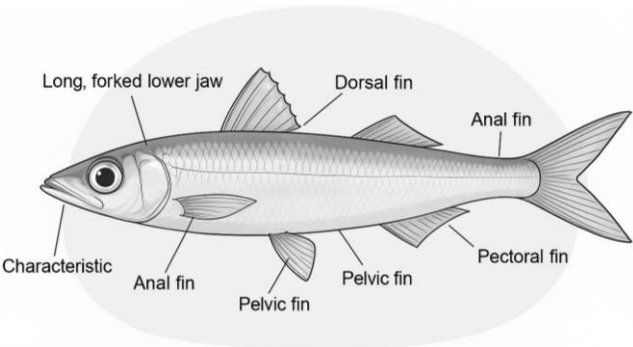


Figure 3. General morphology and anatomical features of anchovies (*Stolephorus* spp.), highlighting diagnostic structures

In many coastal areas, anchovies serve as ecological indicators of ocean productivity and environmental stability. Their population fluctuations are closely linked to oceanographic parameters such as sea surface temperature, upwelling intensity, and chlorophyll-a concentration, making them highly sensitive to climate variability and anthropogenic disturbance (Chaves and Umbria 2003; Lima et al. 2022). For example, El Niño events can drastically reduce anchovy biomass by disrupting upwelling systems and decreasing plankton abundance, as observed in the Pacific Ocean and parts of Southeast Asia. Consequently, long-term monitoring of anchovy populations can provide early warning signs of ecosystem stress or regime shifts in marine environments.

From a conservation perspective, most species within *Stolephorus* remain poorly evaluated, with many listed as Data Deficient (DD) or Least Concern (LC) under the IUCN Red List criteria (Fricke et al. 2019; GBIF 2024). This reflects both their ecological ubiquity and the lack of comprehensive population data, especially for lesserknown or cryptic species. Despite the absence of immediate global extinction risk, anchovy populations face localized threats such as overfishing, habitat degradation (e.g., mangrove and coral reef loss), and marine pollution including microplastics and heavy metals—that can bioaccumulate through trophic transfer (Hasari et al. 2021; Komala et al. 2024).

In response to these pressures, some countries have implemented seasonal fishing quotas, gear restrictions, and Marine Protected Areas (MPAs) to safeguard anchovy stocks and maintain ecosystem integrity. However, the effectiveness of such measures often depends on enforcement capacity and scientific input, including accurate stock assessments and life history data. Additionally, climate change-induced alterations in ocean currents and productivity may require adaptive management strategies that integrate environmental forecasting with fisheries regulation. The summary of their ecological roles and conservation status is presented in the following visual aids (Table 4).

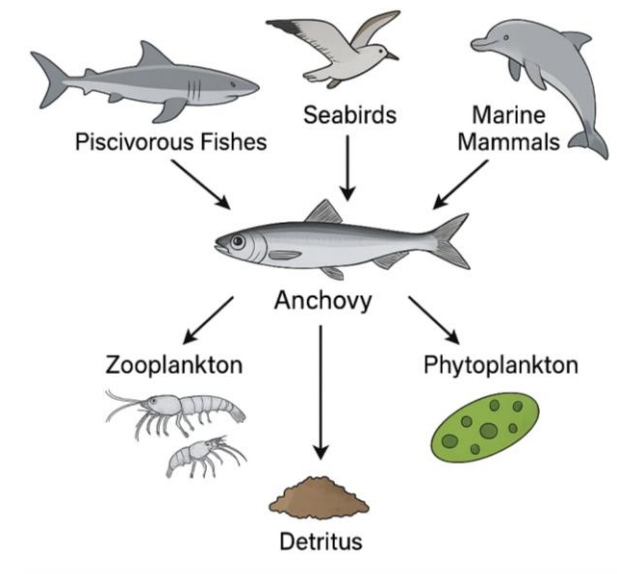


Figure 4. Schematic diagram of anchovy position in the marine trophic food web

Table 4. Reported conservation status of *Stolephorus* species by region

Species	Region	IUCN red list status	Known threats	Data availability
<i>S. indicus</i>	Indian Ocean, Southeast Asia	Least Concern	Overfishing, coastal habitat degradation	Moderate
<i>S. commersonnii</i>	Indo-Pacific, Australia	Data Deficient	Bycatch, marine pollution, and warming temperatures	Limited
<i>S. waitei</i>	Coral reef zones, Northern Australia	Least Concern	Coral degradation, artisanal fisheries	Sparse
<i>S. insularis</i>	Western Indian Ocean	Not Evaluated	Unknown	Very limited
<i>S. dubiosus</i>	Indonesia, Indo-Malay Archipelago	Not Evaluated	Habitat disturbance, taxonomic ambiguity	Very limited

Sources: Fricke et al. (2019); Lima et al. (2022); IUCN (2023); GBIF (2024)

Given their ecological importance and increasing utilization for human consumption, anchovies warrant more rigorous conservation attention. Comprehensive species-level monitoring, coupled with regional collaboration, is essential to prevent future overexploitation and ensure that anchovy populations continue to fulfill their ecological and economic roles in tropical marine systems (Appeltans et al. 2020).

ZOOCHEMICAL ANALYSIS OF ANCHOVIES

Nutritional profile (proteins, fats, minerals, vitamins)

Anchovies (*Stolephorus* spp.) are a nutrient-dense marine food source that offers a well-balanced profile of macronutrients and micronutrients essential for human health. Their small size belies their nutritional richness, as anchovy flesh is particularly high in protein, containing approximately 15-25 g of protein per 100 g of edible portion, depending on species, season, and processing method (Kari et al. 2022). This protein is of high biological value, with a complete amino acid profile that supports tissue maintenance and growth.

The fat content of anchovies varies between 2-10%. It is primarily composed of Polyunsaturated Fatty Acids (PUFAs), especially omega-3 fatty acids such as Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA). These bioactive lipids play crucial roles in reducing cardiovascular risk, regulating inflammation, and supporting neurodevelopment (Barros et al. 2014; Hu et al. 2020). Additionally, anchovies contain cholesterol-lowering

sterols and phospholipids that enhance lipid metabolism and membrane function.

Micronutrient-wise, anchovies are an excellent source of dietary calcium, particularly when consumed with bones, as in dried or fried forms. The calcium content may range from 300 to 1000 mg per 100 g, which significantly contributes to bone health and prevention of osteoporosis, particularly in populations with limited dairy intake. This information is crucial for making informed dietary choices. Iron, zinc, magnesium, selenium, and phosphorus are also abundant, supporting immune response, oxygen transport, and antioxidant defense systems (Chen et al. 2022a). Selenium, in particular, acts as a cofactor for glutathione peroxidase, an important antioxidant enzyme, while zinc is essential for enzymatic activity and DNA repair.

In terms of vitamins, anchovies provide moderate to high levels of fat-soluble vitamins, especially vitamin D and vitamin A. These vitamins play a crucial role in calcium absorption, bone mineralization, and visual function. In addition, anchovies also provide appreciable amounts of water-soluble B vitamins, such as niacin (B3), riboflavin (B2), and vitamin B12, which are also present in appreciable amounts and contribute to energy metabolism and neurological health (Galasso et al. 2020a).

To illustrate the comprehensive nutrient composition of anchovies and their dietary value relative to other small pelagic fish (Table 5). Anchovies' nutritional profile affirms their potential as a low-cost, high-impact food for public health and food security strategies, particularly in coastal and low-resource settings. Their integration into local diets, either fresh or in processed forms, can help combat hidden hunger and contribute to sustainable nutrition solutions.

Table 5. Nutritional composition of anchovies (per 100 g fresh weight)

Nutrient	Anchovies (<i>Stolephorus</i> spp.)	Sardines (<i>Sardinella</i> spp.)	Mackerel (<i>Rastrelliger</i> spp.)	Notes
Protein (g)	18.2-25.0	20.0-24.0	19.5-22.0	High-quality, complete amino acid profile
Fat (g)	4.5-10.0	10.0-15.0	8.0-12.0	Mostly polyunsaturated (omega-3)
Omega-3 (EPA + DHA, g)	1.2-2.5	1.5-3.0	1.8-2.8	Cardioprotective and anti-inflammatory
Calcium (mg)	450-1000	300-500	150-250	Highest when consumed with bones
Iron (mg)	2.0-4.5	1.8-3.5	1.2-2.5	Important for hemoglobin synthesis
Vitamin B12 (µg)	8.5-12.0	9.0-13.0	8.0-11.0	Supports nerve function and metabolism
Vitamin D (IU)	250-600	300-550	200-500	Key for calcium absorption and immunity
Selenium (µg)	30-60	25-55	20-45	Antioxidant cofactor (GPx)

Sources: Barros et al. (2014); Galasso et al. (2020); Kari et al. (2022)

Amino acid composition

The high protein content of anchovies (*Stolephorus* spp.) is complemented by a well-balanced profile of amino acids, both essential and non-essential, which contributes to their high nutritional and functional value. These amino acids not only serve as the building blocks for protein biosynthesis but also participate in physiological processes such as neurotransmission, immune response, antioxidant defense, and metabolic regulation (Andriani and Muhdar 2021; Mutalipassi et al. 2021). The quality of anchovy protein is particularly high due to its digestibility and the presence of all essential amino acids in proportions suitable for human dietary needs.

Among the essential amino acids, anchovies are notably rich in lysine, leucine, isoleucine, methionine, and threonine (Kari et al. 2022). Lysine plays a key role in calcium absorption and collagen formation, while leucine and isoleucine are critical for muscle repair and metabolic signaling (Aggarwal and Bains 2022). Methionine serves as a precursor to sulfur-containing compounds and antioxidants like glutathione (Castro et al. 2023). These amino acids are indispensable in human diets, especially in populations that rely on cereal-based staples with limited amino acid profiles.

In addition, non-essential amino acids such as glutamic acid, alanine, serine, and arginine are abundant in anchovy tissue. Glutamic acid functions as an excitatory neurotransmitter and is a precursor for γ -Aminobutyric Acid (GABA), whereas alanine is involved in energy metabolism and glucose regulation. Arginine supports immune function and nitric oxide synthesis, contributing to vascular health. The interaction between these amino acids contributes to the functional versatility of anchovies as both a food and a potential therapeutic agent (Leke and Schousboe 2016).

The amino acid profile may vary based on environmental factors, including water temperature, food availability, and physiological maturity, as well as postharvest processing methods such as fermentation or hydrolysis. For example, fermented anchovy products have been reported to exhibit enhanced concentrations of free amino acids and bioactive peptides due to proteolysis by endogenous or microbial enzymes (Azfaralariff et al. 2023; Kan et al. 2025). To illustrate the comparative abundance of amino acids in anchovies, Table 6 summarizes typical concentrations reported per 100 g of raw anchovy protein.

This amino acid richness contributes to the growing interest in anchovies as a raw material for bioactive peptide production. Enzymatic hydrolysis of anchovy protein using proteases such as alcalase or papain can yield short-chain peptides with demonstrated antioxidant, antihypertensive, and antimicrobial properties (Shaik and Sarbon 2022a; Kan et al. 2025). Such functional applications further elevate anchovies from traditional protein sources to ingredients of high potential in nutraceutical and pharmaceutical formulations.

Albumin and functional proteins

Albumin is a major water-soluble protein found in anchovy muscle tissue, serving as a critical biomolecule for maintaining osmotic pressure, binding and transporting endogenous and exogenous substances, and preserving structural integrity in both fish and human physiology. In *Stolephorus* spp., albumin content contributes not only to nutritional value but also to several biological activities, including antioxidant, anti-inflammatory, and immunomodulatory functions (Quan et al. 2020; Park et al. 2022). The concentration of albumin in fresh anchovy meat has been reported to range between 1.5 and 3.0% of total crude protein, varying by species and freshness status.

In the context of human health, fish-derived albumin is of particular interest due to its high digestibility and capacity to bind essential micronutrients such as calcium, zinc, and fatty acids. Albumin acts as a carrier protein for poorly water-soluble compounds, including free fatty acids and hormones, making it a functional element in food and pharmaceutical formulations. In anchovies, the presence of albumin is also linked to meat texture and flavor, contributing to consumer acceptability and culinary versatility (Barros et al. 2014).

Extraction of albumin from anchovies can be performed using hot water, salt solutions, or proteolytic enzymes. Among these, thermal extraction (e.g., 60-70°C for 30-60 minutes) is commonly applied to recover albumin in aqueous form for use in the food industry (Park et al. 2022). The isolated albumin can be dried into powder form and utilized in dietary supplements, protein fortifiers, or pharmaceutical carriers. In several studies, albumin-rich extracts from anchovies have demonstrated stability, solubility, and potential bioactivity comparable to bovine serum albumin, suggesting their promise as alternative protein ingredients (Kan et al. 2025).

Table 6. Essential amino acid profile of anchovy protein hydrolysates

Amino acid	Content (mg/100 g edible portion)	Classification	Physiological role
Lysine	1,600-1,800	Essential	Calcium absorption, collagen synthesis
Leucine	1,700-1,900	Essential	Muscle repair, metabolic signaling
Isoleucine	900-1,100	Essential	Hemoglobin formation, energy production
Methionine	700-850	Essential	Antioxidant precursor (glutathione), liver health
Threonine	800-950	Essential	Immune regulation, protein balance
Valine	800-1,000 (estimated, not always reported)	Essential	Muscle metabolism, tissue repair
Phenylalanine	~950-1,050 (in some hydrolysates)	Essential	Precursor for neurotransmitters
Histidine	~600-700 (in growth stages)	Essential (semi)	Growth, tissue repair, and histamine synthesis
Tryptophan	~250-350 (low in fish, variable)	Essential	Serotonin precursor, mood regulation

Sources: Andriani and Muhdar (2021); Kari et al. (2022); Azfaralariff et al. (2023)

Aside from albumin, anchovies also contain other functional proteins such as myosin, actin, tropomyosin, and enzymatic peptides, which contribute to physiological and biochemical properties. These proteins are involved in muscle contraction, post-mortem degradation, and are precursors to bioactive peptides with diverse effects, including antihypertensive and antioxidant activities (Hortillosa et al. 2022; Shaik and Sarbon 2022a). During enzymatic hydrolysis, these structural proteins are broken down into smaller peptides that retain or even enhance biofunctionality, making anchovies an excellent raw material for functional ingredient development. A summary of the main functional proteins and their health-relevant roles is presented in Table 7 and Figure 5.

The functional protein profile of anchovies supports their growing use not only as a staple protein source but also as a platform for bioengineering novel bioactive compounds. Continued research into isolation, characterization, and standardization of these proteins is necessary to unlock their health and industrial potential fully (Musalipassi et al. 2021).

Mineral binding and bioavailability

Anchovies (*Stolephorus* spp.) are excellent natural sources of essential dietary minerals, including calcium, magnesium, iron, zinc, and selenium. Beyond their nutritional abundance, these minerals are bioavailable due to their binding to specific organic molecules such as peptides, proteins (e.g., albumin), and phospholipids within the fish tissue. This mineral-protein interaction facilitates the stability, solubility, and absorption of minerals during digestion, enhancing their functional value in human nutrition (Swastawati et al. 2020; Chen et al. 2022b).

Calcium is the most dominant mineral found in anchovies, especially when consumed with bones (Safitri et al. 2023). It is predominantly bound to collagen and phosphoproteins in the skeletal matrix, allowing for gradual release and absorption during gastrointestinal digestion. Anchovies can provide up to 1,000 mg of calcium per 100 g in dried or whole forms, covering close to the daily recommended intake in many populations with limited dairy consumption. Magnesium and phosphorus are similarly associated with bone and muscle proteins and play synergistic roles in skeletal development and energy metabolism (Lall and Kaushik 2021).

Iron in anchovies is present in both heme and non-heme forms and is typically bound to myoglobin and ferritin

proteins, which enhances its bioavailability relative to plant sources. This makes anchovies a useful dietary component for preventing iron deficiency anemia, particularly in women and children. The zinc content is bound to metalloproteins and enzymes, supporting catalytic activity, immune regulation, and tissue repair. Meanwhile, selenium, often found in the form of selenoproteins, contributes to antioxidant enzyme activity and thyroid function (Galasso et al. 2020a).

These mineral-binding mechanisms are significant because they reduce the likelihood of mineral precipitation in the digestive tract and enhance transport across intestinal membranes. The interaction between proteins and divalent metal ions is regulated by specific amino acid residues such as histidine, cysteine, and glutamic acid that form coordinated covalent bonds with metal ions, stabilizing them in bioavailable forms (Park et al. 2022). This makes anchovy-derived mineral complexes favorable for use in functional food and nutraceutical formulations. To better illustrate the role of anchovies in delivering essential minerals, Table 8 summarizes the key minerals found, their binding forms, and associated physiological functions. The high mineral bioavailability of anchovies, coupled with their affordability and widespread accessibility, supports their inclusion in public health nutrition strategies targeting micronutrient deficiencies. Furthermore, anchovy-based mineral-peptide complexes are being explored as ingredients in fortification programs and therapeutic food products for vulnerable populations.

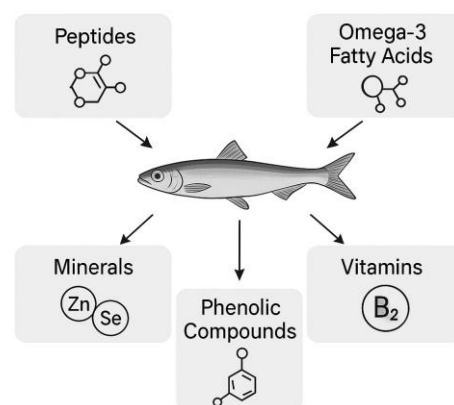


Figure 5. Overview of bioactive compounds identified in anchovy tissues

Table 7. Functional roles of albumin and other anchovy-derived proteins

Protein/peptide	Relative abundance	Functional role	Application potential
Albumin	Moderate (1.5-3%)	Osmoregulation, fatty acid, and micronutrient transport	Nutritional supplements, pharmaceutical carriers
Myosin	High	Muscle contraction, structural integrity	Source of bioactive peptides, texture enhancement
Actin	High	Cytoskeletal framework, elasticity	Functional protein source, peptide precursor
Tropomyosin	Medium	Regulatory protein in muscle contraction	Allergen marker, possible peptide hydrolysate precursor
Protein hydrolysates	Variable	Antioxidant, antihypertensive, and antimicrobial activities	Functional foods, nutraceuticals, therapeutic candidates

Sources: Park et al. (2022); Shaik and Sarbon (2022); Sun et al. (2022)

Enzymatic and hormonal components

Anchovies (*Stolephorus* spp.) contain a variety of endogenous enzymes and hormonal compounds that contribute to both their physiological function as living organisms and their potential value as bioactive food ingredients. These enzymatic components including proteases, lipases, and amylases play central roles in muscle metabolism, digestion, post-mortem autolysis, and preservation of fish freshness. In the context of food processing and biotechnology, such enzymes are also harnessed to produce bioactive peptides through hydrolysis, enhancing the nutraceutical potential of anchovy-derived products (Jeyashakila et al. 2022; Hortillosa et al. 2022).

Proteases, the most abundant among anchovy enzymes, facilitate the breakdown of muscle proteins into peptides and free amino acids. This proteolytic activity is crucial not only for muscle turnover in vivo but also for generating antioxidant, antihypertensive, and antimicrobial peptides during post-harvest fermentation or enzymatic hydrolysis (Shaik and Sarbon 2022a). Lipases, meanwhile, catalyze the hydrolysis of triglycerides into free fatty acids and glycerol, influencing flavor formation and lipid digestion. In anchovy fermentation products such as fish sauce, endogenous lipases contribute to the release of omega-3-rich lipid fractions with recognized health benefits (Kan et al. 2025).

Although less abundant in fish than in herbivorous species, amylases are present in trace amounts in anchovy gut tissue and assist in the partial digestion of glycogen or

starch residues from ingested plankton. These enzymes may also have applications in microbial enzyme production when anchovy tissues are used as substrates in fermentation media (Oktavia et al. 2018).

In addition to enzymes, anchovies possess hormonal substances that regulate metabolic and physiological processes, both within their own systems and as potentially bioactive components in human health. These include thyroid hormones (e.g., thyroxine/T4 and triiodothyronine/T3), steroid precursors, and small amounts of peptide hormones (Nurmawati et al. 2022). Although present in low concentrations, these hormones may exert biological effects after ingestion, particularly when derived from concentrated extracts or fermented preparations.

Thyroid hormones in fish regulate growth, metamorphosis, and basal metabolism. When consumed, these hormones-or their precursors, such as iodine-bound compounds may contribute to thyroid support in iodinedeficient populations. Some steroidal precursors, such as cholesterol derivatives found in anchovies, also serve as substrates for endogenous hormone biosynthesis in the human body. Table 9 summarizes the main enzymatic and hormonal compounds found in anchovies and their known or potential biological significance. Although the direct pharmacological use of anchovy-derived hormones remains limited, understanding their presence opens new avenues for research in marine-based bioactive compounds. Advances in extraction, purification, and delivery systems may enable future development of functional products utilizing these enzymatic and hormonal elements.

Table 8. Bioavailability of minerals and their binding affinity in anchovy tissues

Mineral	Average content (mg/100 g)	Primary binding form	Physiological function
Calcium	450-1000	Bone matrix (collagen, phosphoprotein)	Bone health, muscle contraction
Magnesium	60-120	Complexed with phospholipids	ATP metabolism, neuromuscular function
Iron	2.0-4.5	Ferritin, myoglobin	Oxygen transport, red blood cell formation
Zinc	1.5-3.0	Metalloproteins, enzymes	Enzymatic catalysis, immune regulation
Selenium	30-60 µg	Selenoproteins (e.g., GPx)	Antioxidant defense, thyroid hormone activation
Phosphorus	200-300	Hydroxyapatite, nucleotides	Energy storage (ATP), bone mineralization

Sources: Swastawati et al. (2020); Chen et al. (2022); Kari et al. (2022)

Table 9. Enzymes and hormones identified in anchovy extracts and their physiological roles

Compound type	Specific molecule	Biological role in fish	Functional or health relevance
Protease	Trypsin, pepsin-like enzymes	Muscle turnover, digestion	Generation of antioxidant and ACE-inhibitory peptides
Lipase	Pancreatic-type lipase	Lipid mobilization, flavor development	Facilitates lipid metabolism, improves digestibility
Amylase	α-Amylase (low abundance)	Digestion of glycogen/starch in the gut	Potential in prebiotic fermentation processes
Thyroid hormone	T3, T4	Growth regulation, metabolic rate control	May support thyroid function in iodine-deficient populations
Steroidal precursor	Cholesterol	Hormone biosynthesis	Substrate for steroid and vitamin D synthesis
Peptide hormone	Insulin-like peptides	Glucose regulation	Potential anti-diabetic activity through glycemic control

Sources: Oktavia et al. (2018); Hortillosa et al. (2022); Nurmawati et al. (2022)

BIOLOGICAL ACTIVITIES OF ANCHOVIES

Antioxidant mechanisms

Oxidative stress defined as an imbalance between the production of reactive oxygen species (ROS) and the capacity of biological systems to neutralize them—is a central contributor to cellular damage, aging, and chronic diseases such as cancer, cardiovascular disorders, and neurodegenerative conditions. Anchovies (*Stolephorus* spp.), as marine food resources, offer a promising source of antioxidant compounds that counteract oxidative stress through multiple molecular pathways (Galasso et al. 2020a; Engwa et al. 2022).

The antioxidant potential of anchovies is primarily attributed to three groups of bioactive components: (i) omega-3 polyunsaturated fatty acids (PUFAs), particularly EPA and DHA; (ii) antioxidant peptides generated through enzymatic hydrolysis of anchovy protein; and (iii) trace minerals and vitamins with redox-regulating functions, including selenium, zinc, vitamin E, and vitamin D (Hasari et al. 2021; Sun et al. 2022). These compounds operate both independently and synergistically to neutralize free radicals, chelate pro-oxidant metals, and modulate intracellular antioxidant enzyme systems such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT).

Antioxidant peptides, typically consisting of 2-20 amino acids, are liberated from anchovy muscle proteins through enzymatic hydrolysis using enzymes such as alcalase, papain, or pepsin. These peptides contain amino acid residues such as histidine, tyrosine, cysteine, or methionine that donate electrons or hydrogen atoms to neutralize ROS (Shaik and Sarbon 2022a) (Figure 6). In vitro studies using DPPH and ABTS assays have demonstrated that anchovy protein hydrolysates exhibit high radical-scavenging activity, comparable to that of synthetic antioxidants like BHA or tocopherol under certain conditions (Kan et al. 2025).

Omega-3 fatty acids, while not classical antioxidants, exert indirect antioxidant effects by modulating inflammatory signaling and enhancing the activity of cellular antioxidant defenses. EPA and DHA have been shown to activate Nrf2 signaling pathways, which induce the expression of endogenous antioxidant enzymes. They also inhibit the production of pro-inflammatory cytokines and lipid peroxidation products that would otherwise promote oxidative tissue damage (Yang et al. 2020) (Figure 7).

The contribution of micronutrients is also notable. Selenium, present in anchovies as selenoproteins, plays an essential role in the catalytic function of GPx, which reduces hydrogen peroxide and lipid hydroperoxides. Zinc stabilizes cell membranes and modulates metallothionein expression, which scavenges hydroxyl radicals. Vitamin E (tocopherols) interrupts lipid peroxidation chains in membranes, while vitamin D may contribute to redox homeostasis through immunomodulatory functions (Barros et al. 2014). The mechanisms and sources of antioxidant activity in anchovies are summarized in Table 10.

The multifunctionality of anchovy-derived antioxidants makes them suitable candidates for inclusion in functional foods, dietary supplements, and pharmaceutical preparations targeting oxidative stress-related disorders. Future research should focus on standardizing hydrolysis protocols, identifying specific antioxidant peptide sequences, and conducting clinical trials to validate efficacy *in vivo*.

Antimicrobial and anti-inflammatory effects

Anchovies (*Stolephorus* spp.) exhibit significant antimicrobial and anti-inflammatory activities, primarily due to the presence of bioactive peptides, unsaturated fatty acids, and trace elements that interfere with microbial viability and inflammatory signaling pathways. These bioactivities are particularly evident in anchovy protein hydrolysates and fermented anchovy products, which contain concentrated peptides with specific biological functions (Shaik and Sarbon 2022a; Kan et al. 2025).

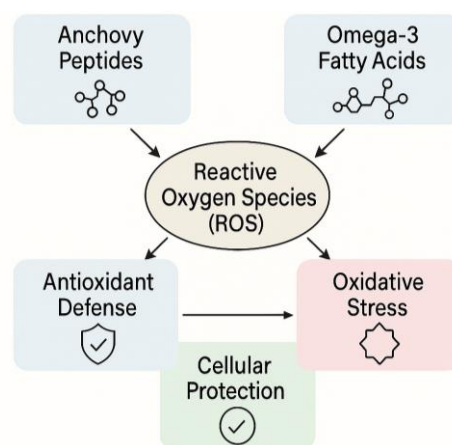


Figure 6. Mechanism of antioxidant activity of anchovy peptides and omega-3 fatty acids

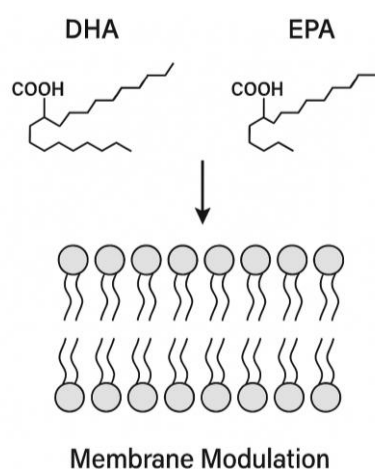


Figure 7. Molecular structure of DHA and EPA and their role in membrane modulation

Table 10. Antioxidant assays and efficacy values of anchovy-derived compounds

Component type	Source in anchovy	Assay method	Reported activity / efficacy	Mechanism of action
Antioxidant peptides	Protein hydrolysates (enzymatic)	DPPH, ABTS	70-90% radical scavenging at 1-5 mg/mL	Electron donation, metal chelation
Omega-3 fatty acids	Muscle lipids (EPA, DHA)	Lipid peroxidation assay (TBARS)	40-60% inhibition of MDA formation in vitro	Membrane stabilization, Nrf2 activation
Selenium	Selenoproteins (GPx precursor)	GPx enzyme activity	Increased GPx activity in supplemented models	Reduces hydroperoxides, supports redox balance
Zinc	Metalloproteins	SOD activity assay	Upregulated SOD expression in tissue cultures	Free radical scavenging, enzyme cofactor
Vitamin E	Lipid fractions	FRAP, ORAC	Comparable to α -tocopherol in lipid-soluble extracts	Breaks the lipid peroxidation chain
Vitamin D	Fat-soluble matrix	Cellular antioxidant assay	Modulates oxidative stress indirectly via immune regulation	Hormone-mediated antioxidant defense

Sources: Galasso et al. (2020); Hasari et al. (2021); Shaik and Sarbon (2022); Sun et al. (2022);

Antimicrobial activity is primarily attributed to small peptides (<10 kDa) released during enzymatic hydrolysis using proteases such as pepsin, trypsin, or alcalase. These peptides often possess amphipathic structures and cationic charges that enable them to bind to negatively charged bacterial membranes, causing disruption, pore formation, and eventual cell lysis (Hortillosa et al. 2022). Studies have shown that anchovy-derived peptides exhibit inhibitory effects against a diverse range of Gram-positive and Gram-negative bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica*, and *Listeria monocytogenes*. This broad-spectrum antimicrobial activity is a testament to the potential of these peptides as natural food preservatives or as leads for antimicrobial drug development.

Fermented anchovy products, such as fish sauce, have also demonstrated significant antimicrobial effects. This is due to the presence of low-molecular-weight peptides, organic acids, and high salt concentrations that effectively inhibit microbial growth. Additionally, anchovy lipids—particularly omega-3 fatty acids—are known to exert bacteriostatic or bactericidal effects by disrupting microbial membranes and suppressing the synthesis of proinflammatory lipopolysaccharides in pathogenic strains (Park et al. 2022).

On the anti-inflammatory front, anchovy-derived compounds modulate inflammatory responses through both direct and indirect mechanisms. Omega-3 fatty acids (EPA and DHA) are precursors to specialized pro-resolving mediators (SPMs) such as resolvins and protectins, which actively terminate inflammation by inhibiting neutrophil infiltration, reducing cytokine production (e.g., TNF- α , IL6), and promoting macrophage-mediated clearance of inflammatory debris (Yang et al. 2020) (Figure 8). These effects are further enhanced by selenium and zinc, which stabilize cellular redox status and downregulate NF- κ B-mediated pro-inflammatory gene expression.

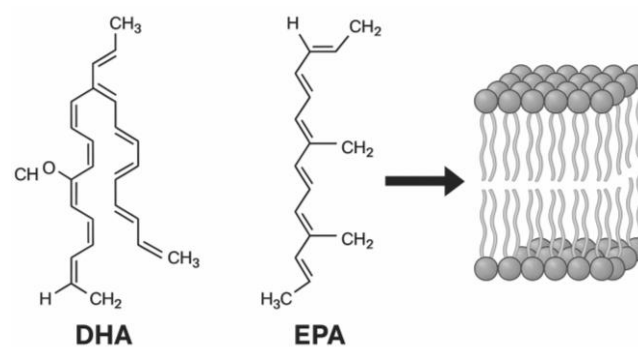
Protein hydrolysates from anchovies have also been shown to reduce nitric oxide (NO) production and prostaglandin E₂ (PGE₂) synthesis in lipopolysaccharide-stimulated macrophage models. These actions reflect the immunomodulatory potential of specific peptide sequences

that interfere with inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2) expression, two key mediators in chronic inflammatory conditions (Shaik and Sarbon 2022a). A summary of these effects is presented in Table 11.

The dual antimicrobial and anti-inflammatory actions of anchovy-derived compounds highlight their relevance in developing functional foods, nutraceuticals, and even natural therapeutics. Their potential to reduce reliance on synthetic antibiotics and anti-inflammatory drugs warrants further exploration, including clinical validation and formulation into safe, bioavailable products.

Antithrombotic and cardiovascular benefits

Cardiovascular Diseases (CVDs) remain the leading cause of global mortality, largely driven by a combination of thrombosis, dyslipidemia, inflammation, and oxidative stress. Anchovies (*Stolephorus* spp.) contain a suite of bioactive compounds most notably omega-3 fatty acids, peptides, and minerals such as selenium that collectively contribute to cardioprotective effects, including antithrombotic, anti-inflammatory, and lipid-modulating properties (Galasso et al. 2020a; Yang et al. 2020).

**Figure 8.** Pathways of antimicrobial and anti-inflammatory effects of anchovy peptides

The antithrombotic potential of anchovies is primarily linked to the high content of long-chain omega-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids competitively inhibit arachidonic acid metabolism, leading to the production of thromboxane A3 (TXA3) a weak platelet aggregator and vasoconstrictor instead of thromboxane A2 (TXA2), which strongly promotes platelet aggregation and vasoconstriction. By shifting the eicosanoid balance, omega-3s from anchovies reduce platelet activation and blood clot formation (Barros et al. 2014).

Additionally, EPA and DHA are precursors of resolvins, specialized pro-resolving lipid mediators that not only limit neutrophil infiltration in vascular tissue but also promote endothelial healing and vasodilation. These compounds work synergistically with selenium, which functions as a cofactor for Glutathione Peroxidase (GPx), reducing lipid peroxidation in vascular endothelium and thus preserving vessel integrity (Chen et al. 2022a).

Protein hydrolysates from anchovies have also demonstrated promising cardiovascular effects. Certain peptides exhibit Angiotensin-Converting Enzyme (ACE) inhibitory activity, thus contributing to blood pressure

reduction. These peptides mimic the action of pharmacological ACE inhibitors, preventing the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor, and promoting vasodilation through bradykinin preservation (Shaik and Sarbon 2022a).

Moreover, anchovy consumption is associated with improved lipid profiles. Studies indicate that regular intake of small pelagic fish rich in omega-3s can lower serum triglycerides, increase HDL cholesterol, and reduce LDL oxidation all of which contribute to reduced risk of atherosclerosis and myocardial infarction. Anchovy-derived phospholipids also support lipid transport and membrane fluidity, important for maintaining cardiovascular homeostasis (Park et al. 2022).

Table 12 summarizes the cardiovascular and antithrombotic benefits of anchovy-derived bioactive compounds. The multifaceted cardiovascular benefits of anchovies make them a valuable component in heart-healthy diets. Their bioactive compounds act on multiple levels from endothelial protection to platelet function regulation offering both preventive and adjunctive potential for CVD management. Future work should focus on peptide isolation, clinical trials, and formulation into targeted nutraceuticals.

Table 11. Antimicrobial and anti-inflammatory bioactivities of anchovy peptides

Bioactive component	Source or derivation	Target / mechanism	Observed bioactivity	Application potential
Antimicrobial peptides	Enzymatic protein hydrolysates	Disrupt bacterial membranes, inhibit metabolism	Inhibitory effect against <i>E. coli</i> , <i>S. aureus</i> , <i>Salmonella</i>	Natural preservatives, antimicrobial nutraceuticals
Omega-3 fatty acids	Muscle lipids (EPA, DHA)	Bacterial membrane disruption, LPS suppression	Bacteriostatic effects; synergy with peptides	Immunonutrition, infection control
Organic acids	Fermented anchovy products	Low pH, microbial metabolic inhibition	Inhibits spoilage and pathogenic bacteria	Traditional food preservation
Selenium & zinc	Muscle-bound trace minerals	NF- κ B inhibition, redox stabilization	Downregulates pro-inflammatory cytokines (TNF- α , IL-6)	Anti-inflammatory support
Anti-inflammatory peptides	Peptide fractions from hydrolysis	Suppress iNOS and COX-2, inhibit PGE2 and NO production	Reduced inflammation in macrophages and LPS-induced models	Functional food for inflammatory diseases
Resolvin precursors	EPA and DHA	Formation of SPMs (resolvins, protectins)	Promotes resolution of inflammation and tissue repair	Chronic inflammation management

Sources: Yang et al. (2020); Hortillosa et al. (2022); Park et al. (2022); Shaik and Sarbon (2022)

Table 12. Cardiovascular and metabolic regulatory effects of anchovy omega-3s

Compound / component	Mechanism of action	Health effect	Target pathway or marker
EPA and DHA (omega-3 PUFAs)	Inhibit thromboxane A2 (TXA2) synthesis, produce TXA3	Antithrombotic, vasodilation	Platelet aggregation, eicosanoid profile
EPA and DHA	Precursor of resolvins and protectins	Resolution of inflammation, endothelial protection	SPM signaling, inflammation markers
ACE-inhibitory peptides	Block conversion of angiotensin I to angiotensin II	Antihypertensive effect	Renin-angiotensin system
Selenium	Cofactor of glutathione peroxidase (GPx)	Endothelial antioxidant protection	Lipid peroxidation (MDA), GPx activity
Phospholipids	Improve lipid transport and membrane fluidity	Elevate HDL, reduce LDL oxidation	Lipid profile (HDL, LDL, TG)
Bioactive peptides	Inhibit DPP-IV and α -glucosidase	Improve insulin secretion, delay glucose absorption	Blood glucose, incretin activity
Zinc	Stabilizes insulin, supports β -cell activity	Enhance insulin synthesis and storage	Pancreatic function
Adiponectin modulation	Upregulated by omega-3s	Improves insulin sensitivity	Adipokine profile

Sources: Galasso et al. (2020); Yang et al. (2020); Chen et al. (2022); Park et al. (2022); Shaik and Sarbon (2022)

Anti-diabetic and metabolic regulation

Metabolic disorders, particularly type 2 diabetes mellitus (T2DM), are characterized by insulin resistance, hyperglycemia, and chronic low-grade inflammation. Increasing attention has been directed toward dietary interventions involving bioactive compounds from marine resources, including anchovies (*Stolephorus* spp.), which offer promising antidiabetic and metabolic regulatory effects through multiple biochemical pathways (Galasso et al. 2020a; Shaik and Sarbon 2022a).

One of the primary antidiabetic components in anchovies is omega-3 polyunsaturated fatty acids (PUFAs), especially EPA and DHA. These fatty acids enhance insulin sensitivity by modulating membrane fluidity, activating peroxisome proliferator-activated receptors (PPARs), and reducing adipose tissue inflammation. EPA and DHA have been shown to downregulate proinflammatory cytokines (e.g., TNF- α , IL-1 β) and upregulate adiponectin a hormone that promotes glucose uptake and fatty acid oxidation thus improving systemic glucose homeostasis (Yang et al. 2020).

Protein hydrolysates from anchovy muscle also exhibit antidiabetic activity, particularly by inhibiting α glucosidase and dipeptidyl peptidase-IV (DPP-IV). α Glucosidase inhibitors delay carbohydrate breakdown and glucose absorption in the intestine, thereby reducing postprandial blood glucose spikes. DPP-IV inhibitors, on the other hand, prolong the half-life of incretin hormones (GLP-1 and GIP), which stimulate insulin secretion and suppress glucagon release (Shaik and Sarbon 2022a). These effects have been validated in vitro and animal models, suggesting anchovy peptides as functional ingredients for glycemic control.

Minerals such as zinc and selenium, abundantly present in anchovies, are critical for insulin synthesis, storage, and secretion. Zinc stabilizes insulin hexamers and contributes to β -cell function, while selenium exerts antioxidant protection for pancreatic tissues through glutathione peroxidase activity (Chen et al. 2022a). The combined effect of these micronutrients supports metabolic regulation and reduces oxidative damage associated with diabetic complications.

Additionally, anchovy intake has been linked to improved lipid metabolism, a crucial factor in managing metabolic syndrome. EPA and DHA reduce hepatic lipogenesis and increase fatty acid β -oxidation via PPAR- α activation. These changes contribute to reduced serum triglycerides and improved lipid profiles, indirectly supporting insulin sensitivity and lowering cardiovascular

risk in diabetic patients (Park et al. 2022). The key metabolic regulatory effects of anchovy-derived components are summarized in Table 13 and Figure 9. These findings highlight anchovies as a functional dietary option for metabolic health, with integrated effects on glucose regulation, insulin signaling, and lipid metabolism. Future research should focus on identifying and purifying the most active peptide fractions and on evaluating their efficacy through human clinical trials.

Anti-cancer and anti-carcinogenic properties

Marine-derived foods are increasingly recognized for their chemopreventive potential, owing to a diversity of bioactive compounds capable of modulating cellular proliferation, oxidative stress, apoptosis, and inflammation. Anchovies (*Stolephorus* spp.) contain multiple classes of such compounds including omega-3 fatty acids, antioxidant peptides, and trace elements that may contribute to anticancer and oral health-promoting (anti-carcinogenic) effects (Galasso et al. 2020a; Shaik and Sarbon 2022a).

Anti-cancer effects

The anticancer potential of anchovy-derived compounds is primarily mediated through apoptosis induction, inhibition of inflammatory signaling, and oxidative stress reduction. EPA and DHA, the dominant omega-3 fatty acids in anchovies, modulate membrane lipid composition and reduce the activity of pro-inflammatory transcription factors such as NF- κ B and STAT3, which are commonly overexpressed in cancer cells. These effects lead to decreased expression of genes involved in cell survival and angiogenesis (e.g., Bcl-2, VEGF) and increased proapoptotic signaling (e.g., Bax, caspases) (Yang et al. 2020) (Figure 10).

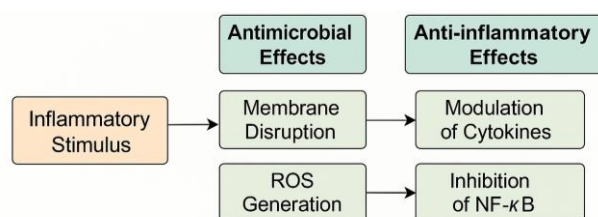


Figure 9. Proposed mechanisms of anchovy-derived compounds in metabolic regulation

Table 13. Anti-diabetic and metabolic regulatory compounds in anchovies (*Stolephorus* spp.)

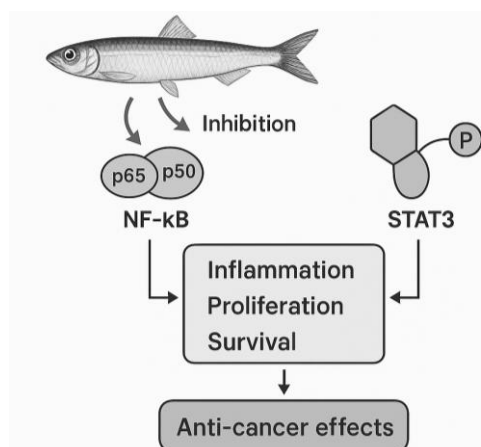
Compound / component	Mechanism of action	Metabolic effect
EPA and DHA (omega-3 PUFAs)	Activate PPARs, reduce inflammation, and increase adiponectin	Enhance insulin sensitivity, reduce glucose levels
Bioactive peptides	Inhibit α -glucosidase and DPP-IV	Delay glucose absorption, increase insulin release
Zinc	Stabilizes insulin, supports β -cell function	Promotes insulin secretion
Selenium	Antioxidant via GPx activity	Protects pancreatic islets
Phospholipids	Improve cell signaling and fat transport	Regulate lipid and glucose metabolism

Sources: Galasso et al. (2020a); Yang et al. (2020); Chen et al. (2022a); Shaik and Sarbon (2022a)

Table 14. Anti-cancer and anti-carcinogenic compounds in anchovies (*Stolephorus* spp.)

Bioactive component	Mechanism of action	Target / effect	Health relevance
EPA and DHA (omega-3)	Inhibit NF- κ B and STAT3 signaling; induce apoptosis	↓ Bcl-2, ↑ caspase activity, ↓ VEGF	Suppress tumor growth, angiogenesis
Antioxidant peptides	Scavenge ROS; disrupt mitochondrial function	↑ Bax/Bak, ↓ cell proliferation	Cytotoxic to cancer cells
Selenium	Incorporated in selenoproteins; modulates apoptosis	↑ DNA repair, ↑ immune surveillance	Reduces oxidative DNA damage, tumor risk
Zinc	Cofactor in DNA repair enzymes and tumor suppressor proteins	↓ Mutation rate, ↑ cell cycle regulation	Maintains genomic stability
Antimicrobial peptides	Disrupt <i>Streptococcus mutans</i> membrane integrity	↓ Plaque formation, ↓ acid production	Prevent dental caries
Protein hydrolysates	Inhibit bacterial metabolism and biofilm formation	↓ <i>S. mutans</i> adhesion, ↓ lactic acid production	Promote oral hygiene and cariostatic effects
Omega-3 fatty acids	Anti-inflammatory action in periodontal tissues	↓ Gingival inflammation, ↑ healing	Support oral and gum health

Sources: Yang et al. (2020); Hortillosa et al. (2022); Sun et al. (2022); Shaik and Sarbon (2022)

**Figure 10.** Anti-cancer mechanisms via NF- κ B/STAT3 inhibition by anchovy bioactives

In vitro studies have shown that anchovy protein hydrolysates can inhibit the proliferation of human colon, breast, and liver cancer cells by arresting the cell cycle and inducing mitochondrial dysfunction. These effects are often linked to specific low-molecular-weight peptides rich in hydrophobic or aromatic amino acids that interact with cancer cell membranes or intracellular targets (Kan et al. 2025).

Additionally, selenium and zinc in anchovies support DNA repair, inhibit tumor growth, and enhance immune surveillance. Selenium, in particular, has been implicated in reducing cancer risk via its incorporation into selenoproteins, which act as antioxidants and modulators of apoptosis (Table 14).

Anti-carcinogenic effects

Anchovies also show promise as functional foods in promoting oral health, especially by exerting anticarcinogenic effects. Antimicrobial peptides and enzymes derived from anchovy muscle or fermented anchovy products (e.g., fish sauce) inhibit the growth of

Streptococcus mutans, a key bacterium responsible for dental plaque formation and tooth decay (Hortillosa et al. 2022).

Omega-3 fatty acids contribute to oral health by reducing gingival inflammation and oxidative stress, while zinc plays a direct role in inhibiting plaque formation and reducing halitosis (Rajaram et al. 2021). Moreover, anchovy-derived protein hydrolysates have been shown to decrease biofilm production in vitro, suggesting their potential application in functional oral care products.

Taken together, the anticancer and anti-carcinogenic properties of anchovies reflect their potential as biofunctional ingredients for both preventive healthcare and oral hygiene applications. These dual benefits underscore the importance of expanding research into specific compound isolation, bioactivity validation, and clinical formulation development (Shaik and Sarbon 2022a).

Structure-function relationship of bioactive compounds

The biological activities exhibited by anchovy-derived compounds are closely related to their molecular structure, particularly the presence of specific functional groups, chain lengths, and conformational properties that determine their interaction with biological targets. Understanding this structure-function relationship is essential for elucidating the mechanisms underlying the observed antioxidant, antimicrobial, anti-inflammatory, and metabolic effects of anchovy-based compounds.

Bioactive peptides, generated through the enzymatic hydrolysis of anchovy proteins, are typically composed of 2 to 20 amino acid residues with distinct physicochemical characteristics (Kari et al. 2022). Their activity is largely governed by the presence of hydrophobic (e.g., leucine, valine), aromatic (e.g., tyrosine, phenylalanine), and sulfur-containing residues (e.g., cysteine, methionine). These side chains facilitate membrane interactions, radical scavenging, or enzyme inhibition depending on their orientation and charge. For instance, the ability of a peptide to scavenge free radicals correlates with the electron-donating potential

of phenolic and sulfur groups. In comparison, peptides with basic residues (e.g., lysine, arginine) are more effective at binding to bacterial membranes due to electrostatic attraction.

The structure of omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is also critical to their function. These long-chain polyunsaturated fatty acids contain multiple cis-double bonds that create kinks in the carbon backbone, enhancing their fluidity and enabling their integration into cellular membranes (Hishikawa et al. 2017). This structural property modulates membrane microdomains, affecting receptor function, signal transduction, and lipid mediator production. Moreover, the position and number of double bonds influence their metabolic conversion into bioactive lipid mediators such as resolvins and protectins, which are responsible for anti-inflammatory and pro-resolving effects (Bannenberg 2009).

Trace elements, such as selenium and zinc, are highly dependent on coordination chemistry and protein-binding specificity for their bioactivity. Selenium exerts its function when incorporated as selenocysteine into the active sites of enzymes like glutathione peroxidase, where the Se-H group serves as a redox-active center. Zinc, meanwhile, stabilizes protein domains (e.g., zinc fingers) or acts as a cofactor for enzymes like Superoxide Dismutase (SOD), with its tetrahedral coordination geometry enabling electron transfer reactions essential for antioxidative processes (Klotz et al. 2003; Wołonciej et al. 2016).

Finally, the spatial conformation of anchovy-derived enzymes and hormones plays a key role in determining their biological function. Structural domains such as the active sites of proteases or the receptor-binding motifs of peptide hormones must maintain precise folding to interact with substrates or cellular receptors. Even slight modifications in tertiary or quaternary structure due to pH, temperature, or hydrolysis can significantly alter bioavailability and efficacy (Kari et al. 2023).

Collectively, the functional diversity of anchovy bioactives stems from a high degree of structural specialization, which enables specific interactions with molecular targets in human physiology. Advances in proteomics, lipidomics, and molecular modeling continue to reveal how these structural attributes translate into potent biological effects, guiding the development of targeted nutraceuticals and functional foods based on anchovy compounds (Giannetto et al. 2016).

ENVIRONMENTAL AND PUBLIC HEALTH CONSIDERATIONS

Heavy metal and microplastic contamination risks

While anchovies (*Stolephorus* spp.) are widely regarded as a nutritious and bioactive-rich food resource, increasing concerns have emerged regarding their contamination by heavy metals and microplastics, particularly in coastal and estuarine environments subject to anthropogenic pollution. As small pelagic fish with high metabolic rates and wide foraging behavior, anchovies may accumulate contaminants both through trophic transfer and direct environmental exposure (Karami et al. 2017; Barhoumi et al. 2022).

Heavy metal accumulation in anchovies is primarily influenced by local water quality, sediment characteristics, and bioavailability of toxic elements such as mercury (Hg), cadmium (Cd), lead (Pb), and arsenic (As) (Table 15). Although anchovies are relatively low on the food chain and thus expected to exhibit lower biomagnification than apex predators, recent studies have detected variable levels of these metals in anchovy tissues depending on the region. For example, anchovy samples from industrialized coastal zones in the Mediterranean, South China Sea, and Indonesian estuaries have shown detectable concentrations of Pb and Cd exceeding WHO/FAO recommended safety thresholds (Syakti et al. 2019; Riani et al. 2024). Chronic exposure to such metals through frequent consumption may pose risks, including neurotoxicity, renal impairment, and carcinogenicity, especially among vulnerable populations such as pregnant women and children.

In parallel, microplastic contamination has emerged as a global threat to marine food safety. Anchovies are known to ingest microplastics defined as plastic fragments <5 mm either directly by mistaking them for plankton or indirectly through contaminated prey. Once ingested, these particles can accumulate in the gastrointestinal tract and, in some cases, translocate to edible tissues. The concern is not only the physical presence of the plastic particles but also their role as vectors for adsorbed persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs), dioxins, and polycyclic aromatic hydrocarbons (PAHs), which may leach into surrounding tissues and elicit endocrine-disrupting, mutagenic, or immune-suppressive effects (Rochman et al. 2015) (Figure 11).

Table 15. Heavy metal concentrations reported in anchovies from selected regions

Region / location	Mercury (Hg) (mg/kg)	Cadmium (Cd) (mg/kg)	Lead (Pb) (mg/kg)	Exceeds FAO/WHO (Limits)	Potential sources
North Coast of Java, Indonesia	0.03-0.11	0.02-0.09	0.15-0.35	Pb often exceeds limit	Urban runoff, industrial effluents
South China Sea	0.05-0.12	0.01-0.06	0.10-0.25	No or occasional exceedance	Shipping, aquaculture discharge
Mediterranean Sea (coastal)	0.07-0.18	0.03-0.10	0.20-0.40	Cd and Pb exceed limits	Industrial zones, historical contamination
Eastern Gulf of Thailand	0.01-0.06	0.01-0.04	0.08-0.20	Within safe limits	Low anthropogenic pressure
Estuaries of Sumatra	0.02-0.08	0.01-0.05	0.12-0.30	Pb near or above limit	Agriculture runoff, mining residue

Note: FAO/WHO Max Limits: Hg = 0.5 mg/kg; Cd = 0.1 mg/kg; Pb = 0.3 mg/kg (wet weight basis). Sources: Syakti et al. (2019); Barhoumi et al. (2022); Riani et al. (2024)

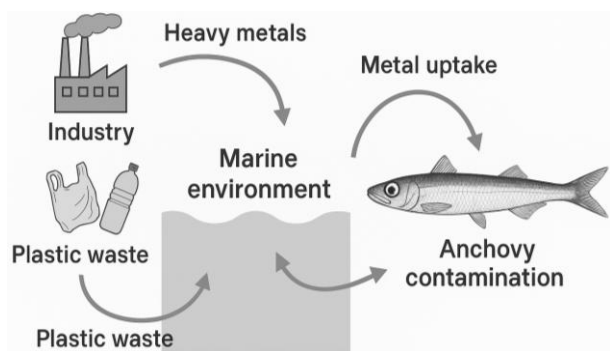


Figure 11. Sources and pathways of heavy metal and microplastic contamination in anchovies

Despite evisceration and cooking reducing some risk, certain forms of anchovy consumption such as whole dried fish, fermented paste, or powder supplements may retain higher contamination potential. These contaminants could include heavy metals like mercury or persistent organic pollutants like dioxins and PCBs. Moreover, bioaccumulated toxins may not be eliminated by traditional preparation methods, necessitating greater attention to source origin and monitoring protocols.

These concerns highlight the urgent need for routine surveillance of contaminant levels in anchovy stocks, especially in fisheries near densely populated or industrialized coastlines. Policy recommendations include tighter regulation of marine pollution sources, certification of seafood safety, and public advisories on maximum intake levels. However, it is crucial to remember that these are not the only solutions. Further studies are required to assess the bioaccessibility and toxicokinetics of contaminants in processed anchovy products and to explore mitigation strategies, such as depuration techniques or bioremediation.

Overexploitation and ecological impacts

As one of the most heavily harvested marine fish groups globally, anchovies (*Stolephorus* spp.) face increasing pressure from overfishing, driven by high demand for both human consumption and use as fishmeal in aquaculture and poultry feed industries. Their biological characteristics such as short lifespan, early maturation, and high fecundity make anchovies resilient to some extent. However, recent trends suggest that intensive and unregulated harvesting is outpacing their natural replenishment, particularly in coastal regions of Southeast Asia, West Africa, and the eastern Mediterranean (FAO 2022; Ali et al. 2025).

The ecological consequences of anchovy overexploitation extend beyond population declines. Anchovies occupy a key trophic position in marine food webs, acting as energy conduits between primary producers (phytoplankton, zooplankton) and higher trophic levels, including commercially important predatory fish, seabirds, and marine mammals. Reductions in anchovy biomass can trigger cascading effects disrupting predator foraging success, altering species interactions, and shifting

ecosystem structure and resilience (Smith et al. 2011). In areas like the Benguela and Humboldt Current systems, where anchovies dominate pelagic fish assemblages, such imbalances have led to regime shifts, with long-term impacts on biodiversity and fishery yields. Compounding these pressures, anchovy habitats particularly estuaries, coral reef edges, and nearshore upwelling zones are increasingly affected by climate change, pollution, and habitat degradation. Temperature anomalies, acidification, and coastal development influence spawning grounds and larval survival, exacerbating recruitment variability. This makes *Stolephorus* spp. particularly vulnerable to multistressor environments, where overfishing interacts with environmental stressors in complex and often unpredictable ways.

Addressing these challenges requires an EcosystemBased Fishery Management (EBFM) approach that integrates anchovy population dynamics, environmental variability, and multi-species interactions. Establishing spatial-temporal fishing regulations, catch limits, and community-based monitoring systems can help maintain sustainable stocks. Additionally, the inclusion of anchovy biomass trends in ecosystem health indicators is vital for marine spatial planning and conservation. Despite their economic and nutritional importance, anchovies are often undervalued in formal fisheries governance frameworks. This underrepresentation leads to data gaps in stock assessment and undermines the enforcement of conservation measures. Strengthening fisheries data collection especially for small-scale and artisanal fisheries along with regional collaboration, is critical to ensuring long-term sustainability.

Sustainable harvesting and management strategies

To safeguard the ecological and economic roles of anchovies (*Stolephorus* spp.) amid increasing exploitation pressures, the implementation of sustainable harvesting and fisheries management strategies is imperative. Given their pivotal role in tropical food webs and coastal economies, these strategies should be informed by biological stock assessments, ecosystem dynamics, and socioeconomic considerations, ensuring both the long-term viability of anchovy populations and continued benefits for coastal communities that depend on them (Salim et al. 2020; FAO 2022) (Figure 12). A foundational approach is the application of science-based catch limits, derived from Maximum Sustainable Yield (MSY) estimates and precautionary reference points. Regular stock assessments incorporating age-structured models, recruitment variability, and environmental indices are essential to prevent overexploitation. In regions with limited data availability, data-poor methodologies such as length-based indicators, Productivity-Susceptibility Analysis (PSA), or empirical harvest control rules can offer interim solutions for management (Froese et al. 2012). Seasonal and spatial fishing closures are effective in protecting anchovy spawning aggregations and juvenile recruitment zones. For instance, several countries have instituted moratoriums during peak breeding months or established Marine Protected Areas (MPAs) along critical nursery habitats.

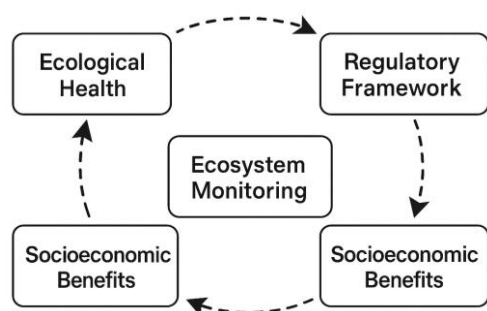


Figure 12. Conceptual framework of ecosystem-based anchovy fishery management, showing a cyclical interaction between ecosystem monitoring, regulatory enforcement, and ecological health. The first Socioeconomic Benefits node (right) refers to direct outcomes such as improved fishery income and local employment following regulatory compliance, while the second Socioeconomic Benefits node (bottom left) denotes long-term benefits including livelihood sustainability, food security, and social equity, driven by enhanced ecosystem and policy feedback

These measures support population recovery while minimizing ecological disruption, especially when designed with local fisher participation and compliance monitoring. The success of such interventions often depends on community trust, socio-cultural alignment, and enforceability. The integration of Ecosystem-Based Fisheries Management (EBFM) principles is also key. EBFM acknowledges the role of anchovies as mid-trophic connectors and seeks to balance their exploitation with the needs of dependent predators and ecosystem services. Such approaches could include multi-species stock modeling, habitat preservation, and food-web-based catch allocation strategies, especially in ecologically sensitive systems like coral reef margins and coastal upwelling zones (Pikitch et al. 2004). Moreover, cross-sector collaboration linking fisheries, conservation, and coastal development agendas is needed to operationalize EBFM effectively.

In many regions, anchovy fisheries are dominated by small-scale and artisanal operations, which pose both challenges and opportunities for sustainability. While these fisheries may have lower environmental footprints, they often lack institutional support, data recording, and access to sustainable technologies. Participatory governance and inclusive decision-making platforms, along with capacity building, co-management frameworks, and inclusion of Traditional Ecological Knowledge (TEK), can enhance stewardship and accountability in these sectors.

Emerging innovations such as selective fishing gear, low-impact harvest methods, and real-time monitoring systems can further reduce bycatch, habitat disturbance, and Illegal, Unreported, and Unregulated (IUU) fishing. Technological tools like electronic logbooks, machine learning for stock prediction, mobile applications for catch reporting, and satellite-based vessel tracking are gaining traction in community-based fisheries governance.

Lastly, promoting market-based incentives such as ecolabeling (e.g., MSC certification), fair trade agreements, and value-added product development can align economic interests with sustainability goals. By fostering traceable

supply chains and rewarding responsible practices, these mechanisms encourage fishers and processors to adopt conservation-minded approaches. Consumer education campaigns and retailer partnerships can amplify these incentives and drive demand for sustainably sourced anchovies. Collectively, these strategies underscore the need for integrated, adaptive, and participatory management systems that reflect ecological realities and social contexts. Anchovies, as fast-growing and high-value species, offer an ideal test case for advancing holistic marine resource management in the face of global change. Their management must remain dynamic, evidence-driven, and inclusive to ensure long-term ecological and socioeconomic resilience.

APPLICATIONS AND FUTURE PERSPECTIVES

Functional food and nutraceutical potentials

The increasing recognition of the health-promoting properties of marine bioresources has propelled anchovies (*Stolephorus* spp.) into the spotlight as a promising candidate for functional food and nutraceutical development. Rich in bioactive peptides, omega-3 fatty acids, essential minerals, and antioxidant compounds, anchovies offer multifunctional health benefits that align with current consumer demands for natural, preventive, and therapeutic dietary products (Galasso et al. 2020a; Shaik and Sarbon 2022a).

Anchovies, with their high content of EPA and DHA, are versatile ingredients for a range of functional foods, which are defined as conventional food products that provide additional physiological benefits beyond basic nutrition. These bioactive compounds contribute to cardiovascular protection, neurodevelopment, and antiinflammatory regulation, making anchovies a key ingredient in formulations aimed at reducing noncommunicable disease risk. The potential applications of anchovies in products such as anchovy-enriched spreads, soups, sauces, crackers, and extruded snacks have been explored, with promising results in terms of sensory properties and improved lipid profiles (Park et al. 2022).

Simultaneously, the development of nutraceuticals bioactive compounds delivered in concentrated form (e.g., capsules, tablets, powders) is rapidly gaining momentum. The increasing consumer demand for health-promoting products drives this trend. Anchovy protein hydrolysates, with their peptide-based bioactivities, are particularly attractive for this market. These bioactivities include antioxidant, antihypertensive, and glucose-regulatory effects. These hydrolysates can be obtained through enzymatic processing using food-grade proteases, yielding fractions with low allergenicity and high digestibility. Their potential applications include anti-fatigue supplements, blood sugar regulators, and muscle recovery agents, especially for aging and physically active populations (Chalamaiah et al. 2012).

Fish oils extracted from anchovies are already well-established in commercial omega-3 supplement markets. However, innovations now focus on microencapsulation

techniques, which improve oxidative stability, bioavailability, and controlled release of fatty acids in the gastrointestinal tract. Additionally, fermented anchovy products such as fish sauce and pastes offer natural alternatives with added value as probiotic carriers or mineral supplements. Anchovies also serve as a sustainable and scalable source of functional biomolecules, given their high reproductive rate and availability in tropical and subtropical marine waters. This makes them suitable for long-term supply in health-based product pipelines, potentially leading to economic benefits for the industry. Moreover, the low trophic level and short lifespan of anchovies reduce ecological risks and contamination compared to large pelagic species, offering a safer raw material base (Alfio et al. 2021).

Challenges in this domain include ensuring standardization, quality control, and the utmost regulatory compliance for anchovy-derived products. Variability in bioactive content due to species, habitat, and processing methods must be addressed through rigorous characterization and labeling. Collaboration between fisheries, food technologists, and health scientists is needed to fully exploit the functional potential of anchovies while ensuring safety and consumer confidence. As the global trend toward personalized nutrition and marine-based therapeutics accelerates, *Stolephorus* spp. stand out as a biocompatible, accessible, and multifunctional marine resource for the next generation of health-supporting products (Mutalipassi et al. 2024).

Technological innovations in extraction and processing

The valorization of anchovies (*Stolephorus* spp.) as a source of high-value bioactive compounds has driven significant progress in technological innovations related to extraction and processing methods. Advancements in this area are essential not only to increase yield and purity of target compounds but also to preserve their bioactivity, ensure safety, and enable their application in food, pharmaceutical, and nutraceutical industries (Shaik and Sarbon 2022a; Kan et al. 2025) (Table 16, Figure 13).

Conventional extraction techniques, such as solvent extraction or mechanical pressing, are limited by low

selectivity, thermal degradation, and the use of potentially toxic solvents. To overcome these limitations, modern techniques have been adopted, with enzymatic hydrolysis emerging as the most promising method for isolating bioactive peptides from anchovy proteins. This technique employs food-grade proteases (e.g., alcalase, papain, flavourzyme) under controlled pH and temperature conditions, allowing for the release of functional peptides with antioxidant, antihypertensive, and anti-diabetic properties while maintaining nutritional quality (Galasso et al. 2020a).

Further refinement is achieved through ultrafiltration and membrane fractionation, which enable size-based separation of peptide fractions (<3 kDa, 3-10 kDa, etc.), each with distinct bioactivities. These technologies enhance bioactive concentration, reduce allergenic potential, and allow for precise formulation of nutraceutical blends. In the lipid domain, Supercritical Fluid Extraction (SFE) using CO₂ has attracted attention as an environmentally friendly, solvent-free method for isolating omega-3-rich oils from anchovy tissues. SFE offers advantages in purity, oxidation resistance, and scalability, producing EPA/DHA-rich extracts suitable for encapsulation or direct inclusion in functional foods (Zhou and Wei 2023).

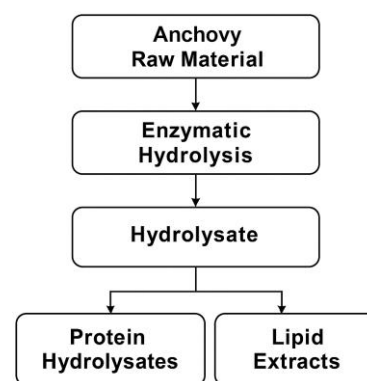


Figure 13. Processing flow of anchovy protein hydrolysates and lipid extracts

Table 16. Technological methods for anchovy bioactive extraction and their advantages

Method	Target compound	Key advantages	Limitations
Enzymatic hydrolysis	Bioactive peptides	High specificity, low temperature, retains bioactivity	Cost of enzymes, time-intensive
Ultrafiltration/membrane tech	Peptide fractionation	Size-selective separation improves bioactivity concentration	Membrane fouling, operational complexity
Supercritical CO ₂ extraction	Omega-3 fatty acids (EPA, DHA)	Solvent-free, high purity, low oxidation risk	High equipment cost, limited for hydrophilic
Spray drying (microencapsulation)	Omega-3s, peptides	Enhances stability, shelf life, and controlled release	May reduce yield or encapsulation efficiency
Controlled fermentation	Peptides, organic acids	Traditional method, natural flavor, microbial enzyme action	Variability in composition, long fermentation
Solvent extraction (conventional)	Total lipids, fatty acids	Simple and widely used	Risk of solvent residues, low selectivity

Note: Galasso et al. (2020); Shaik and Sarbon (2022); Sun et al. (2022)

Additionally, microencapsulation technologies such as spray drying, coacervation, and liposomal entrapment are increasingly being applied to protect sensitive fatty acids and peptides during storage and digestion, improving bioavailability and consumer acceptability. Fermentation-based approaches, traditionally used in Southeast Asian anchovy products like fish sauce and *bagoong*, have been optimized using starter cultures and controlled fermentation systems. These innovations improve consistency, enhance flavor profiles, and may enrich products with bioactive amino acids, peptides, and beneficial microbes. Moreover, integrated biorefinery models are being explored to utilize anchovy by-products such as heads, bones, and viscera for the recovery of gelatin, collagen peptides, enzymes (e.g., trypsin), and calcium-rich materials. These by-products can be processed into functional ingredients for the cosmetic, pharmaceutical, or animal feed industries, supporting circular economy principles and reducing waste (Perry and McClements 2020).

Emerging technologies such as Pulsed Electric Field (PEF) and Ultrasound-Assisted Extraction (UAE) are also under investigation to improve cell disruption and mass transfer efficiency. These technologies could potentially reduce processing time and energy consumption while preserving thermolabile compounds. These innovations collectively contribute to the sustainable utilization of anchovies as a multifunctional marine resource, enabling the development of high-quality, bioactive-rich products for diverse markets. Ongoing research is needed to scale these technologies economically and to assess their environmental impacts, particularly in small-scale and artisanal fishery contexts where infrastructure remains limited (Figure 14).

Research gaps and recommended directions

Despite growing interest in the health benefits and bioactive potential of anchovies (*Stolephorus* spp.), several critical research gaps remain that limit their full scientific validation and commercial exploitation. One of the most pressing needs is for more in vivo or clinical trials to confirm the efficacy and safety of anchovy-derived compounds in human systems. This is crucial for translating laboratory findings into standardized functional food or pharmaceutical products, making the research highly relevant and necessary.

A second major gap concerns the species-specific characterization of bioactives. This is a crucial area of studies that fail to distinguish between different *Stolephorus* species, despite likely variation in biochemical profiles due to genetics, habitat, diet, and seasonal dynamics. Establishing detailed comparative analyses across multiple species and geographic origins is not just essential for quality control, regulatory approval, and targeted product development, underscoring the significance and impact of the research.

The mechanistic understanding of how specific anchovy-derived compounds exert their biological effects particularly antioxidant, anti-inflammatory, anti-diabetic,

and anti-cancer actions also remains limited. While general pathways such as inhibition of oxidative stress or modulation of signaling cascades (e.g., NF- κ B, PPAR) have been proposed, more refined studies using molecular docking, omics-based platforms (proteomics, metabolomics), and systems biology are needed to clarify compound-target interactions.

Additionally, extraction, hydrolysis, and bioactivity testing protocols lack standardization, making cross-study comparison difficult. The development of validated methods for measuring peptide composition, antioxidant activity, enzymatic inhibition, and toxicity is critical for building a coherent body of evidence and ensuring replicability. Environmental and safety assessments also warrant further investigation. Anchovies are vulnerable to heavy metal and microplastic contamination in polluted coastal waters, yet few studies assess the bioaccessibility, accumulation, and toxicokinetics of such contaminants in processed anchovy products. Integrated approaches combining food science with environmental toxicology will be necessary to ensure consumer safety and guide source selection.

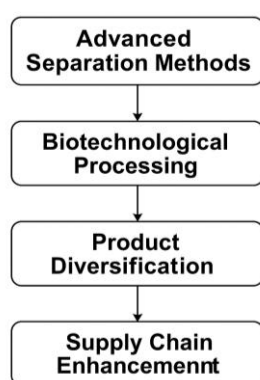
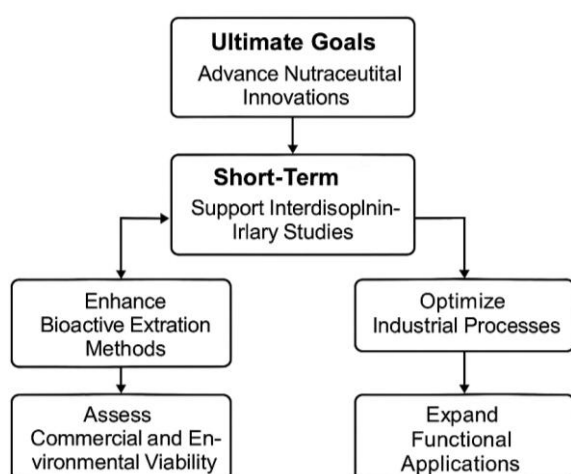
Moreover, the valorization of anchovy processing by-products (e.g., heads, viscera, bones) for bioactive extraction remains underexplored. If processing technologies and market frameworks are adequately developed, these fractions could provide low-cost, sustainable inputs for gelatin, collagen, mineral, or enzyme production, supporting circular bioeconomy goals. Lastly, the integration of local and indigenous knowledge, particularly from regions with long traditions of anchovy use (e.g., Southeast Asia, the Mediterranean), offers valuable insights into preparation methods, consumption patterns, and ethnonutritional relevance areas currently underrepresented in scientific literature. To address these gaps, future research should prioritize clinical trials that validate the health claims of anchovy-derived nutraceuticals, alongside species-specific chemoprofiling using multi-omics platforms to capture biochemical diversity across different *Stolephorus* taxa.

Emphasis should also be placed on mechanistic studies that elucidate the links between molecular structure and physiological function, as well as the development of standardized, scalable, and environmentally friendly extraction methods to ensure consistency and sustainability. In parallel, environmental monitoring and toxicological assessments are needed to evaluate contamination risks associated with heavy metals and microplastics in anchovy-based products.

Finally, greater attention should be given to the valorization of by-products and integration of traditional ecological knowledge, particularly in regions with long-standing cultural uses of anchovies. Collectively, these research directions will enhance the scientific credibility, safety, and real-world applicability of anchovies as a strategic marine bioresource for sustainable nutrition and health promotion (Table 17, Figure 15).

Table 17. Summary of research gaps and proposed scientific directions

Identified research gap	Recommended scientific direction	Expected impact
Lack of clinical validation of health claims	Conduct human trials on anchovy-derived nutraceuticals	Evidence-based support for therapeutic applications
Limited chemoprofiling at the species level	Apply omics-based techniques (e.g., proteomics, lipidomics, metabolomics)	Improved specificity and bioactive standardization
Poor understanding of molecular mechanisms	Perform mechanistic studies linking compound structure to physiological effect	Rational design of functional formulations
Non-standardized extraction and processing methods	Develop green, scalable, and standardized protocols	Increased industrial applicability and reproducibility
Potential environmental contamination in anchovy products	Integrate contaminant monitoring and risk assessment in product development	Enhanced safety and consumer confidence
Underutilized anchovy by-products and traditional knowledge	Explore bioactive recovery from waste and local processing wisdom	Circular bioeconomy and cultural value preservation

**Figure 14.** Technological innovations in anchovy extraction and the value chain development scheme**Figure 15.** Research roadmap for future development of anchovy-based bioresources

CONCLUDING REMARKS

Anchovies (*Stolephorus* spp.) represent a highly valuable marine resource at the intersection of biodiversity, nutrition, and biomedicine. This review has highlighted the broad spectrum of zoochemical compounds present in

anchovies including omega-3 fatty acids, bioactive peptides, essential minerals, and functional proteins—that contribute to various biological activities such as antioxidant, antimicrobial, anti-inflammatory, metabolic, and anti-cancer effects. These bioactives exhibit promising potential for development into functional foods, nutraceuticals, and pharmaceutical agents, supported by emerging evidence from both in vitro and in vivo studies. Ecologically, anchovies play a pivotal role in marine food webs, yet they are increasingly threatened by overexploitation, habitat degradation, and contamination from heavy metals and microplastics. These environmental challenges necessitate integrated strategies for sustainable harvesting, technological innovation, and contamination risk mitigation.

Advances in enzymatic extraction, microencapsulation, and biorefinery approaches have begun to unlock the commercial and therapeutic potential of anchovy-derived products, although further standardization and validation are urgently needed. Despite significant progress, key research gaps remain, particularly regarding species-specific biochemical profiling, mechanistic elucidation, clinical efficacy, and safe consumption thresholds. Addressing these issues through interdisciplinary research will strengthen the foundation for future applications and policy development. Anchovies, due to their global availability, nutritional richness, and functional versatility, are uniquely positioned to contribute to marine-based solutions for health, sustainability, and food security in the coming decades.

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