VOLUME 11 ISSUE 2 2025

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 11, No. 2, 1255-1263 (2025)

International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

Editor-in-Chief: Prof. Ajai Kumar Srivastav

Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

A Comparative Study on Biochemical Analysis and Antimicrobial Activity of Epidermal Mucus of Two Freshwater Fish Species (*Channa punctata* and *Cyprinus carpio*)

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Received: 15th June, 2025; **Accepted:** 22nd November, 2025; **Published online:** 27th December, 2025

https://doi.org/10.33745/ijzi.2025.v11i02.125

Abstract: Freshwater fish possesses mucous glands on their epidermis which produces thick, slimy and sometimes watery mucous. Mucous serves as a protective film to prevent infection by different environmental pathogens, owing to its rich composition of bioactive compounds. This study evaluated the biochemistry of mucous secreted by the two indigenious fish species namely, *Channa punctata* and *Cyprinus carpio* as well as their efficacy against microbial infection,. Mucus samples were collected under sterile conditions and analyzed for protein, carbohydrate and lipid contents. Results showed higher protein concentrations in both species, with *C. carpio* exhibiting 0.951±0.269 mg/ml and *C. punctata* 0.535±0.162 mg/ml. Carbohydrate and lipid contents were also higher in case of *C. carpio* as compared to *C. punctata*. Antimicrobial activity was assessed against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi* using the well diffusion method. Both species displayed inhibitory effects, with *C. carpio* showing greater antimicrobial potential, especially against *S. aureus* (zone of inhibition of 6.15±0.052 mm). The results underscore the defensive role of mucus produced by fishes from their epidermis and suggest its potential as a source of natural antimicrobial agents.

Keywords: Antimicrobial activity, Antimicrobial peptide, Biochemical analysis, *Channa punctata*, *Cyprinus carpio*, Epidermal mucus, Zone of inhibition

Citation: Gogoi Himadri, Sarmah Manash Pratim, Mahanta Bhagyashree, Choudhury Kamal and Shireen Tahsina: A comparative study on biochemical analysis and antimicrobial activity of epidermal mucus of two freshwater fish species (*Channa punctata* and *Cyprinus carpio*). Intern. J. Zool. Invest. 11(2): 1255-1263, 2025.

https://doi.org/10.33745/ijzi.2025.v11i02.125

Introduction

The epithelial surface of fish is enveloped by a slick and slimy layer formed by mucous which acts as both a physical and chemical shield against pathogens (Sajorne and Mabuhay-Omar, 2020). This mucus, secreted by fish, forms a barrier between their internal and external environments, functioning as a lubricant with a mechanical protective system (Hussain and Sachan, 2023). Mucus is a versatile substance that is crucial for communication, breathing, disease resistance, excretion, ionic and osmotic balance and feeding. It contains bioactive metabolites that are vital for the defense mechanism (Dash *et al.*, 2018). The mucus layer is an inherent part of the immune system of fish, comprising phagocytes and natural killer cells. Fish epidermis contains three types of mucus-secreting cells: goblet cells, sacciform cells and club cells (Hilles *et al.*, 2019).

Fish mucus acts as a crucial shield, forming a protective layer against both living and non-living elements in their environment. This mucus is composed of both cellular and humoral components. The cellular aspect includes a mucus membrane and the connective tissue beneath it, while the humoral component comprises some other particles beyond the cellular level found in the mucus produced by epidermis (Sarmah *et al*., 2025). Fish mucus is made up of proteins, lipids, carbohydrates and metabolites. Key proteins and enzymes in the epidermal mucus include antimicrobial peptides (AMPs), proteases, lysozyme, lectins, complement proteins, immuno-globulin, C-reactive proteins (CRP), transferrins, etc. Mucins, which are high molecular weight glycoproteins, give the mucus its viscoelastic and rheological characteristics (Abrami *et al*., 2024). The region of North-East India, particularly Assam and Meghalaya, boasts a rich diversity of freshwater fishes. Among these, the spotted snakehead (*Channa punctata*) and common carp (*Cyprinus carpio*) are native species. These fish typically inhabit ponds, swamps and brackish water systems. In India, these two species are highly valued as food fish due to their taste and consumer preference. The aquatic environments where these fishes reside are often infested with potentially harmful microorganisms. To combat these microbes, the fish have developed adaptive traits, including the abundant secretion of mucus on their skin, which helps them to make a shield over their body against various microbes (Bashir *et al*., 2015; Khan *et al*., 2019; Hossain *et al*., 2020; Bakshi and Rai, 2023). Therefore, this study aims to examine certain biochemical parameters and the efficacy of the mucous secreted by *Channa punctata* and *Cyprinus carpio* against different pathogenic microbes.

Materials and Methods

Experimental animals

The freshwater fish species chosen for this study were *Channa punctata* and *Cyprinus carpio*. These species were selected due to their prevalence in the freshwater bodies of Assam and Meghalaya and their high consumption rates. *C. punctata* naturally inhabits swamps, ponds and brackish water systems and is classified as Least Concern by the IUCN, as there are no significant threats to its population. This species is primarily carnivorous, feeding on crustaceans, mollusks, insects, small fish, semi-digested materials and occasionally plants (Bhuiyan *et al*., 2006). In contrast, *C. carpio* is omnivorous, consuming mollusks, insects, crustaceans and seeds. They are generally live in the benthic zone of water body but also occasionally found in middle zone and surface zone of water body for feeding purpose. Their habitat includes water bodies undergone eutrophication with a muddy benthic zone and water body with condensed macrophytes and microphytes (Badiou *et al*., 2011).

Collection and management of experimental animals

Fresh samples of *C. punctata* and *C. carpio* were obtained from the local market in Beltola, Guwahati, Assam, India weighing approximately 270 g and 1200 g, respectively (Figs. 1a, b). Live specimens collected from the market were brought to the laboratory. The fish specimens

a

b

c

Fig. 1: (a) *Channa punctata*; (b) *Cyprinus carpio*; (c) Management of fishes in laboratory.

were kept in a large container. To maintain water quality and hygiene, the container was filled to one-quarter capacity with water, which was changed every other day (Fig. 1c). The health of the fishes was monitored daily. During the acclimation period, the fishes were fed daily with healthy fish feed by mixing rice bran and wheat flour in a concentration of 4%/kg b wt.

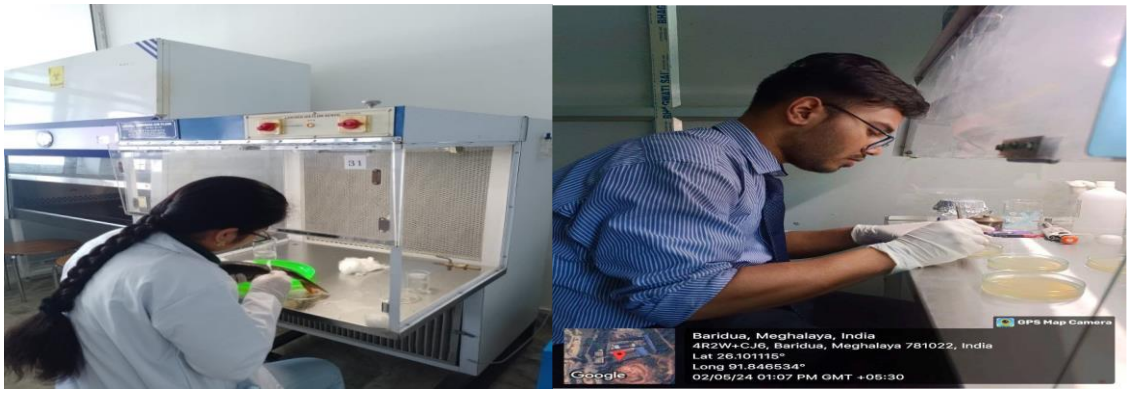
Collection of epidermal mucus

Using a spatula, glass slide, specimen tray, petri dish and dropper sterilized with 70% ethanol, mucus was collected from the two fish specimens. All materials were initially placed in a laminar airflow. The fishes were also washed with 0.85% NaCl. The specimens were then placed on a tray under laminar airflow. After some time, approximately 10 ml of mucus was collected from each fish species from their skin. The mucus was carefully removed by using a disinfected scrapper and a glass slide, from the skin belonging to the dorsal side of the fish (Fig. 2a). The mucous was not collected from the ventral side because due to intestinal contamination the mucous might be contaminated. For the antimicrobial study, physiological saline (0.85% NaCl) was mixed with collected skin mucous in 1:1 ratio and mixed well. After centrifugation for 13 min (5000 rpm) the pallet was collected. After this process, the supernatant was extracted and transferred to a centrifuge tube. The resulting extracts from the two specimens were then stored at 4ºC for later use.

Estimation of total protein in epidermal mucus

Purification of protein from epidermal mucus

To estimate the total protein content, the protein was purified from the mucus using an ethanol precipitation method. Initially, the collected mucus was diluted with water in a 1:2 ratio. This mixture was then stirred at room temperature using a vortex mixer. Following agitation, the mixture was centrifuged at approximately 14000 rpm for 30 min. After centrifugation, the precipitate was discarded, and the supernatant was extracted and cooled with absolute ethanol



a **b**

Fig. 2: (a) Showing collection of epidermal mucus of *C. punctata* and *C. carpio* under laminar flow hood, (b) showing performance of antimicrobial assay under laminar flow hood.

approximately at -20ºC for 20 min. Subsequently, the supernatant was precipitated by adding three volumes of the chilled ethanol, with the process conducted at -20ºC for 2 h. The resulting precipitate was collected through centrifugation at 7200 rpm for 30 min and then stored at 4ºC. The final precipitate was dissolved in 1 ml of 0.1N NaOH, which served as the aliquot samples for protein estimation in the skin mucus of two fish specimens.

Estimation of protein from skin mucus

Protein estimation from skin mucus was performed using the method by Lowry *et al*. (1951). The purified protein sample from the mucus was treated as the unknown, while various concentrations of BSA were used as standards. Optical densities (OD) were measured with a colorimeter, and the results were determined by plotting the OD on a graph.

Estimation of total carbohydrates in epidermal mucus

For estimating the total carbohydrate content in the epidermal mucus of fish specimens, the Anthrone test was employed. Concentrated H_2SO_4 was used to dehydrate carbohydrates, leading to the formation of furfural. Anthranol, the enol tautomer of anthrone, acts as the active reagent in this process. It condenses with the furfural derivative of the carbohydrate, producing a green color in dilute solutions and a blue color in concentrated solutions. The intensity of this color was measured using colorimeter, with the blue-green solution showing maximum absorption at a wavelength of 620 nm.

Lipid analysis in epidermal mucus

Free fatty acid assay was used to analyse lipid content of the mucus. For this procedure, solutions of phenolphthalein indicator and 0.1N NaOH were prepared. To make the phenolphthalein indicator solution, 2 g of phenolphthalein powder were dissolved in 100 ml of ethanol, and the mixture was allowed to cool. Once cooled, final volume was made up to 250 ml by adding distilled water. In preparing the sample, 5 ml of skin mucus from a fish specimen were collected into a conical flask. In another conical flask, 50 ml of absolute ethanol was mixed with a few drops of phenolphthalein indicator. This mixture was neutralized by gradually adding 0.1N NaOH until a light pink hue appeared. The neutralized ethanol was then poured into the flask with the mucus sample, and the solution was mixed thoroughly. The mixture was heated for about 5-10 min until it reached a boil. After cooling, a few drops of phenolphthalein indicator were added. A burette filled with 0.1N NaOH was used to titrate the solution with vigorous stirring until it turned a pale whitish-pink, indicating the endpoint of the titration. For determination of free fatty acids in the mucus

sample, the acid value was first calculated, and by this result free fatty acid content was determined (in percentage or grams) in the sample.

Antimicrobial activity test of epidermal mucus

The well diffusion method was used to evaluate the antimicrobial properties of the epidermal mucus from two fish species, *Channa punctata* and *Cyprinus carpio*, against three bacterial species. Freshly extracted mucus specimens were used for the antimicrobial test.

Inoculation of bacteria

For testing the antimicrobial activity of the mucus samples, three bacteria were chosen: *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi*. These bacterial strains were obtained from the laboratory of Department of Applied Biology, University of Science and Technology, Meghalaya, India. Nutrient broth was prepared for bacterial inoculation. After autoclaving, the inoculation of these three bacteria was carried out under laminar air flow. Following inoculation, the conical flasks were incubated for 24 h at 37ºC to allow bacterial growth.

Antimicrobial activity test

The antimicrobial tests were performed using the well diffusion method. Antimicrobial activity was evaluated using Mueller-Hinton Agar (MHA). To avoid contamination, autoclaving was done for the agar and the equipment like petri dishes, spreader, etc. that were used for the activities. The entire antimicrobial test process was carried out in a laminar air flow. The developing broth culture was spread out on the MHA surface using sterile swab sticks and a spreader (Fig. 2b). Using a sterile cork borer, wells were made in each petri dish, and the holes were filled with roughly 100 µl of freshly collected samples. Ampicillin was used at a dosage of 0.5 mg/ml of sterilized distilled water as a positive control against harmful bacteria. For 24 h, the bacterial cultures were kept at 37ºC. After incubation, a ruler was used to measure the diameter of the inhibition zones surrounding the wells, and the results were recorded in millimeters (mm).

Statistical analysis

Data analysis was done using basic statistical methods such as the mean, standard errorand Student's t-test.

Results and Discussion

Biochemical analysis of epidermal mucus

The main component of the mucus of both fish species was found to be protein. Our results exhibited that the skin mucus of *Channa punctata* and *Cyprinus carpio* had total protein concentrations of 0.535±0.162 mg/ml and 0.951±0.269 mg/ml, respectively. Another significant ingredient in the fishes' epidermal mucus was carbohydrates. According to our findings, *C. punctata* and *C. carpio* mucus contained 0.065±0.004 mg/ml and 0.086±0.003 mg/ml of total carbohydrates, respectively.

The estimation of free fatty acids revealed that the skin mucus of both fish species contains a detectable amount of lipids. When a standardized alkaline solution was added, the color changed from deep pink to light pink, indicating the presence of lipids. According to our findings, each 5 ml sample of mucus from *C. punctata* and *C. carpio* had a free fatty acid concentration of 0.026±0.003% and 0.037±0.003%, respectively.

Proteins make up a larger proportion of the epidermal mucus of both fish species than the lipids and carbohydrates (Table 1). Proteins and carbohydrates were found to be more prevalent in the epidermal mucus of these fish species, while lipids were present in the least amount. The epidermal mucus of *C. carpio* contained 0.951±0.269 mg/ml of proteins, 0.086±0.003 mg/ml of carbohydrates, and 0.037±0.003% of free fatty acid. The protein content of epidermal mucus of *C. punctata* was 0.535±0.162 mg/ml, the carbohydrate content was 0.065±0.004 mg/ml, and the lipids or free fatty acid content was 0.026±0.003% of the sample. Student's t test was conducted between the 2 fish species and significant result was obtained in free fatty acid analysis. Rest of the result was insignificant.

Table 1: Estimation of biochemical components present in epidermal mucus of *C. punctata* and *C. carpio*

Mucus sample of fish	**Total protein concentration** (in mg/ml)	**Total carbohydrate concentration** (in mg/ml)	**Free fatty acidconcentration** (in %)
Channa punctata	0.535±0.162	0.065±0.004	0.026±0.003*
Cyprinus carpio	0.951±0.269	0.086±0.003	0.037±0.003*

Values are mean±SEM; n=3; *indicates p≤ 0.05

Table 2: Zones of Inhibition (ZOI) of epidermal mucus of *C. punctata* and *C. carpio* against three bacterial strains, with ampicillin used as a positive control

Bacterial strains	**Zones of inhibition (in mm)**		
	Channa punctata	***Cyprinus carpio***	**Ampicillin** (0.5 mg/ml)
E. coli	2.13±0.035	3.31±0.053	7.64±0.154
S. aureus	3.60±0.012*	6.15±0.052	4.34±0.164*
S. typhi	2.20±0.140	5.78±0.073*	6.10±0.041

Values are mean±SEM; n=3; *indicates p≤ 0.05

Evaluation of antimicrobial effect of skin mucous

The antimicrobial activities of the epidermal mucus of *C. punctata* and *C. carpio*were observed against three different bacterial strains, namely *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi*. The zone of inhibition (ZOI) was calculated for the antibacterial activities of epidermal mucus of both the fishes.

The epidermal mucus of *Channa punctata* and *Cyprinus carpio* showed different zones of inhibition against the different bacteria (Table 2). The mucus of *C. punctata* showed zones of inhibition of 2.13±0.035 mm, 3.67±0.012 mm and 2.20±0.140 mm against the *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi*, respectively; whereas the skin mucus of *C. carpio* showed zones of inhibition of 3.31±0.053 mm, 6.15±0.052 mm and 5.78±0.073 mm against the *E. coli*, *S. aureus* and *S. typhi*, respectively. The Ampicillin was used as a positive control that showed their activity against all different bacterial stains. The zones of inhibition 7.64±0.154 mm, 4.34±0.164 mm and 6.10±0.041 mm were observed for ampicillin against *E. coli*, *S. aureus* and *S. typhi*, respectively (Table 2). The result obtained from two fish species against 3 bacterial strains were compared with the ampicillin treated group and significant result was found against *S. aureus* by *C. punctata* skin mucous. On the other hand, significant result was obtained against *S. typhi* by *C. carpio* skin mucous. The result against *E. coli* was insignificant for both fish mucus. Figure 3 represents the comparison of the antimicrobial activity of both mucus sample of *C. punctata* and *C. carpio* with respect to three different bacteria. *Cyprinus carpio* showed highest activity than the sample of *Channa punctata* against the three different bacteria. Both the epidermal mucus of *Cyprinus carpio* and *Channa punctata* showed highest activity against *S. aureus* as compared to other two bacterial strains. Tests of significance (t-test) have been done for all data analyses.

a **b** **c**

Fig. 3: Zones of inhibition (ZOI) of epidermal mucus of *C. punctata* and *C. carpio* against (a) *E. coli*, (b) *S. aureus* and (c) *S. typhi* bacterial strains, with ampicillin used as a positive control.

Abbas *et al.* (2020) found different protein in mucus of various major carps native to India and China. The experimental fish species are *Ctenophryngodon idella*, *Hypophthalmichthys moiltrix*, *Labeo rohita*, *Cirrhinus mrigala*, *Catla catla* and *Cyprinus carpio* accordingly and in all fish species they have evaluated the presence of different protein as well as the hemagglutination titer value and alkaline phosphatase activity present in their mucus .They found the protein content in the mucus of *C. carpio* as 1.80±0.09 mg/ml. Also, Asfar *et al.* (2014) worked on the water content, protein content, albumin protein content, fat content, etc. on mucus of Snakehead fish. In the present study, the result obtained from epidermal mucus of *Cyprinus carpio* showed that the concentration of total protein in mucus is 0.951±0.269 mg/ml (Table 1). A biochemical analysis of mucus extracts conducted by Ali *et al*. (2023) revealed that the protein content was higher in samples from *Labeo rohita*, *Ctenopharyngodon idella*, and *Gibelion catla* compared to those from *Hypophthalmichthys molitrix* and *Cirrhinus mrigala*. They also found that carbohydrate and lipid concentrations followed a similar pattern, with lower levels in the latter two species. In the present study, the result obtained from epidermal mucus of *Channa punctata* and *Cyprinus carpio* indicated that the concentration of total proteins in mucus is higher as compared to carbohydrates and lipids. The protein content in the epidermal mucus of *C. punctata* was 0.535±0.162 mg/ml, carbohydrate content was 0.065±0.004 mg/ml and the lipids or free fatty acid content was 0.026±0.003% of sample; whereas the epidermal mucus of *C. carpio* contained 0.951±0.269 mg/ml of proteins, 0.086±0.003 mg/ml of carbohydrate and 0.037± 0.003% of free fatty acid of sample (Table 1).

Kumari *et al.* (2011) investigated mucus of the fresh water fishes *Rita rita* and *Channa punctata* to observe its antimicrobial activity. They observed the antimicrobial activity of mucus against five bacterial stains, such as *Staphyllococus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi.* The zone of inhibition showed by the mucus of *Channa punctatus* against the *S. aureus* and *M. luteus* are 8.75±1.06 mm and 10.0±1.58 mm, respectively. Bhatnagar and Budhalia (2022) conducted studies on epidermal mucus of three Indian major carps to investigate their biochemical components, immune characteristics and its antimicrobial activity. They observed *C. catla*, *L. rohita* and *C. mrigala* and found its composition protein, carbohydrate, lipid etc. The zones of inhibition

were 14.53±0.088 mm, 14.16±0.120 mm and 14.43±0.088 mm, respectively. In the present study, the mucus of *C. punctata* showed zones of inhibition of 2.13±0.035 mm, 3.67±0.012 mm and 2.20±0.140 mm against the *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi*, respectively; whereas the skin mucus of *C. carpio* showed zones of inhibition of 3.31±0.053 mm, 6.15±0.052 mm and 5.78±0.073 mm against the *E. coli*, *S. aureus* and *S. typhi*, respectively. It was also found that *Cyprinus carpio* showed highest activity than the sample of *Channa punctata* against the three different bacteria. The epidermal mucus of *Cyprinus carpio* and *Channa punctata* showed highest activity against *S. aureus* as compared to other two bacterial strains (Table 2).

Conclusion

The current investigation revealed that the mucus of two fish species i.e., *Channa punctata* and *Cyprinus carpio* is very effective to reduce bacterial growth against three tested bacterial strains namely *E. coli*, *S. aureus* and *S. typhi.* With this we can conclude that the skin mucus may help both the organisms to protect their skin from many pathogenic bacteria when they are in their natural aquatic habitat. The biochemical analysis showed a very good presence of protein content, which may indicate the presence of many antimicrobial peptides in the epidermal mucous of both fishes.

Ethical Statement

No animals have been used or sacrificed for this study, hence Ethical Approval not required.

Author Contributions

Choudhury Kamal and *Mahanta Bhagyashree*: Conceptualization; *Gogoi Himadri* and *Sarmah Manash Pratim*: Data Curation and Formal Analysis; *Sarmah Manash Pratim* and *Gogoi Himadri*: Resources; *Shireen Tahsina*: Visualization; *Gogoi Himadri* and *Sarmah Manash Pratim*: Writing Original draft; *Sarmah Manash Pratim*: Writing, Review and Editing; *Mahanta Bhagyashree*: Supervision. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

Authors are thankful to the authorities of University of Science and Technology, Meghalaya for providing facilities for carrying out this experiment. Authors are also thankful to the laboratory staff and the fish supplier for their kind help.

Conflict of Interest

The authors declare no conflicts of interest.

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