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Research Article

Biochemical Analysis and Antimicrobial Activity of Epidermal Mucus of Freshwater Mud Eel *Monopterus cuchia*

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ABSTRACT

Background: Among the fishes of North-East India, especially Assam, the *Monopterus cuchia* is unique for its habitat in the mud. The freshwater mud eel *M. cuchia* inhabits various shallow, vegetation-rich freshwater environments, including ponds, canals, rivers, beels and flood plains. They thrive abundantly in mud holes in shallow beels with low oxygen levels. The entire winter season is spent by this fish species in mud and exposed to many soil bacteria. To avoid microbial invading this species produces epidermal mucous. **Materials and Methods:** In the present study, the biochemical analysis of the epidermal mucus of *M. cuchia* was done through estimation of total protein, total carbohydrates and analysis of lipids by adopting appropriate protocols. The antimicrobial activity of epidermal mucus in various concentrations of 100, 60, 40, 30 and 10% were observed against two different bacteria by well diffusion method. **Results:** Biochemical characterization and antimicrobial potential of epidermal mucus extracts of *M. cuchia* showed that carbohydrate concentration was higher followed by protein and lipid content. Among proteins, carbohydrates, and lipids, the skin mucus of fish contained the smallest amount of lipids. The antimicrobial activity assay against *Escherichia coli* and *Staphylococcus aureus* revealed that the maximum inhibition zone was observed with 100% epidermal mucus concentration for both bacterial strains. This result was comparable to the effectiveness of ampicillin, which served as the positive control in the experiment. **Conclusion:** These findings indicate that the skin mucus of *M. cuchia* may serve as a rich source of novel antimicrobial agents for potential use in treatments related to fish and human health.

Keywords: Antimicrobial activity, Antimicrobial peptide, Biochemical analysis, Epidermal mucus, *Monopterus cuchia*, Zone of inhibition.

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INTRODUCTION

Fish in aquatic environments constantly encounter various pathogenic microorganisms, necessitating complex defense mechanisms for their survival. The initial line of defense against a broad spectrum of pathogens is provided by the epidermal mucus and its components.[1] Secreted by epidermal goblet cells, this mucus layer consists of water and glycoproteins.[2] Fish mucosa comprises both cellular and humoral elements. The cellular component includes the mucous membrane and its underlying connective tissue, while the humoral part encompasses extracellular molecules present in the skin mucus.[3] The epidermal mucus contains numerous crucial proteins and enzymes, including proteases, Antimicrobial Peptides (AMPs), lectins, immunoglobulin, lysozyme, complement proteins, transferrins and various other antibacterial proteins and peptides. These components play a vital role in the innate immunity of fish.[4]

North-East India, especially Assam is rich with its enormous freshwater fish diversity. Among the other fishes, the *M. cuchia* is an indigenous species of Northeast India and is unique for its habitat in the mud. The entire winter season is spent by this fish species in mud. So, they witness a large variety of soil dwelling microbes. To challenge with such microbes *M. cuchia* adopt certain adaptive characteristics among which copious secretion of mucus on their skin is a protective skill against soil microbes and to avoid desiccation. In the past many studies has been conducted on the context of antimicrobial potential of epidermal mucus of *Monopteus albus* and the results were very promising[5-7] but the study on *M. cuchia* is very little. Therefore, in the present study, certain biochemical parameters and antimicrobial activity were conducted on epidermal mucus of freshwater mud eel *M. cuchia*.

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MATERIALS AND METHODS

Experimental animal

Monopterus cuchia was formerly referred as *Amphipnous cuchia* (Hamilton, 1822). Shallow, well-vegetated freshwater ecosystems, such as ponds, canals, rivers, beels, and flood plains, serve as habitats for the *M. cuchia*. They inhabit plenty of mud holes in shallow beels with low oxygen content. The entire winter season is spent by this fish species in mud. The *M. cuchia* is a carnivorous and nocturnal animal and feed on foods like small fishes, molluscs and worms etc.

Collection of experimental animal

The fresh fish sample of *M. cuchia* was collected from Six-mile fish market, Guwahati, Assam. Its weight was about 410 g (Figure 1a). Live specimen of *M. cuchia* that was collected from the local market kept into the laboratory. The fish specimen was maintained in a big container. To preserve water quality and maintain hygienic conditions, the container was filled to one-quarter capacity with water, which was replaced every other day. The fish's health was monitored daily.

Collection of epidermal mucus

With the help of spatula, glass slide, specimen tray, petridish, dropper, sterilized by 70% ethanol mucus was collected. All the materials were kept in laminar air flow first. The fish were also washed with 0.85% NaCl. Then the specimen was kept on a tray under laminar air flow. Following a period, approximately 10 mL of mucus secreted on the fish's epidermal surface was gathered as a sample. Using a sterile spatula and glass slide, the mucus was carefully removed from the dorsal area of the body (Figure 1b). To prevent intestinal contamination, no mucus was collected from the ventral region. For the antimicrobial study, the collected mucus samples were thoroughly combined with an equal volume of sterilized physiological saline (0.85% NaCl).

The suspended precipitate was separated through centrifugation, which was performed at 5000 rpm for duration of 15 min. Following this process, the supernatant was extracted and transferred to a centrifuge tube. The resulting extract was then kept at 4°C for subsequent use.

Estimation of total protein in epidermal mucus

Purification of protein from epidermal mucus of M. cuchia

For estimation of total protein, purification of protein from the mucus was done by using precipitation method with ethanol. To prepare the sample, the collected mucus was diluted with twice its volume of water. The resulting mixture was then agitated at an ambient temperature using a vortex mixer. After stirring, the mixture was centrifuged at about 14000 rpm for 30 min. After centrifuged, the precipitate was removed. The supernatant was extracted and subsequently chilled along with absolute ethanol at -20°C for 20 min. Following this, the supernatant underwent precipitation using 3 volumes of the cooled ethanol. This precipitation process was carried out at -20°C for 2 hr. The resulting precipitation was then gathered through centrifugation at 7200 rpm for 30 min, after which it was kept at 4°C for storage. The final amount of precipitate was dissolved in 1 mL of 0.1N NaOH which will be the aliquot sample for estimation of protein in skin mucus of *M. cuchia*.

Estimation of protein from skin mucus

The estimation of protein was done by Lowry *et al.*, (1951) method. The purified protein sample from the mucus was used as unknown and different concentration of BSA was used as slandered. Optical Densities (OD) were taken using colorimeter and then the result was calculated by plotting the OD in graph.

Estimation of total carbohydrates in epidermal mucus

The anthrone test estimated carbohydrate content. Concentrated H_2SO_4 is used to dehydrate carbohydrates, resulting in the formation of furfural. The active reagent in this process is anthranol, which is the enol tautomer of anthrone. This compound 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 can be measured using colorimetry. The blue-green solution exhibits a maximum absorption at a wavelength of 620 nm.

Lipid analysis in epidermal mucus

A free fatty acid assay was used to conduct the lipid analysis. For this test, phenolphthalein indicator solution and 0.1N NaOH solution were prepared. To prepare the phenolphthalein indicator solution, 2 g of phenolphthalein powder was mixed with 100 mL of ethanol until fully dissolved. To create a 0.1N NaOH solution, 1 gram of Sodium Hydroxide pellets was mixed with 225 mL of distilled water. The mixture was then left to cool down. After cooling, the final volume of this solution was made to 250 mL by adding more distilled water. In the sample preparation, 5 g of skin mucus of *M. cuchia* was weighted in a conical flask with the help of weighing machine. In a separate conical flask, 50 mL of absolute ethanol was combined with a few drops of phenolphthalein indicator. This mixture was then neutralized by carefully adding 0.1N NaOH until a faint pink color appeared. The neutralization process was necessary for ethanol. The neutralized ethanol was subsequently added to the flask containing the mucus sample, and the solution was thoroughly mixed. The mixture was heated on a hot plate for approximately 5-10 min until it boiled. After allowing the mixture to cool, a few drops of phenolphthalein indicator were added. A burette was filled with 0.1N NaOH, and the titration began with vigorous stirring. The process continued until the solution in the conical flask turned a pale whitish-pink color, signaling the titration's endpoint. For the determination of

free fatty acids in a sample of skin mucus of fish, the acid value was calculated first and then the calculated acid value was used to find out the free fatty acid (in percentage or grams) in the sample.

Antimicrobial activity test of epidermal mucus

The well diffusion technique was employed to assess the antimicrobial efficacy of the epidermal mucus of *M. cuchia* against two bacterial species. For conducting the antimicrobial examination, a newly extracted mucus specimen was utilized.

Inoculation of bacteria

For the test of antimicrobial activity of the mucus sample, two (2) bacteria were selected; these are *Escherichia coli* and *Staphylococcus aureus*. These bacteria were collected from the laboratory of Applied Biology department, USTM.

Nutrient broth was prepared for the inoculation of bacteria. After keeping them in autoclave, inoculation done of these two bacteria under laminar air flow. After inoculation was done the conical flask was kept in the incubator for 24 hr at 37°C for growth of the bacteria.

Antimicrobial activity test

The well diffusion method was employed to conduct antimicrobial tests. For the antimicrobial test, Mueller-Hinton Agar (MHA) was prepared. To avoid contamination, safety measures like autoclaving are done for the agar and the equipment like petri dishes, spreader, etc. that were used for the activities. The mucus of *M. cuchia* was prepared in several dilutions: 100%, 60%, 40%, 30%, and 10%. Undiluted mucus was considered to have a 100% concentration, while the other concentrations were achieved by diluting with distilled water using pipetting methods. The whole procedure of antimicrobial test was done in the laminar air flow (Figure 1c). Using sterile swab sticks and spreader, the broth culture which was growing straked on MHA surface. In culture media, wells were punched in each petri dish using a sterile cork borer and about 100µL of freshly collected samples of various concentrations were used to fill the holes. As a positive

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Figure 1: a. Showing *M. cuchia,* b. Showing a collection of epidermal mucus from *M. cuchia* under laminar flow hood, c. Showing performance of antimicrobial assay under laminar flow hood.

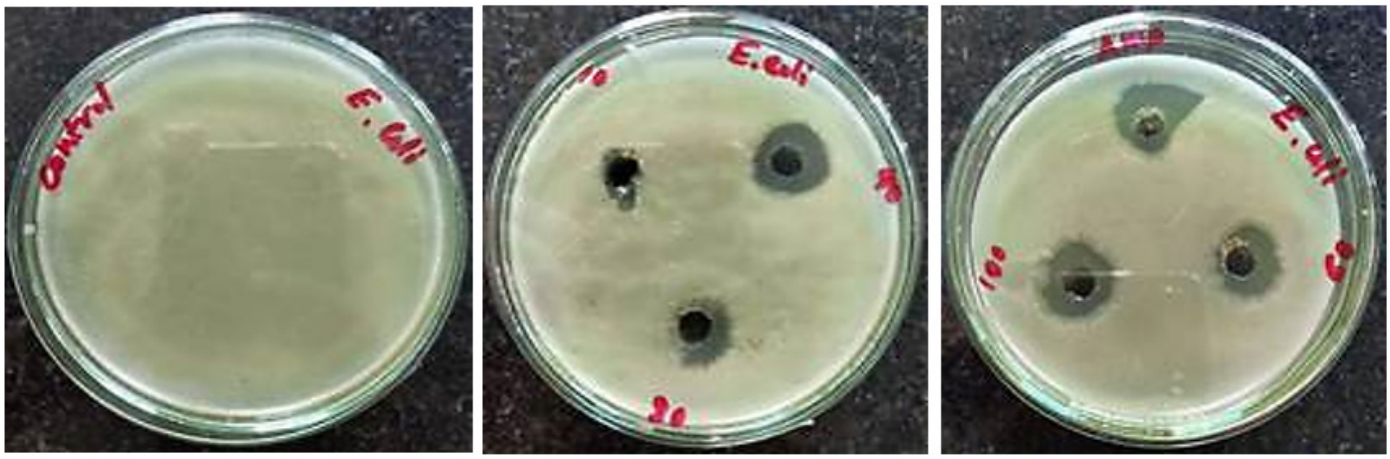

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Figure 2: Showing Zones of Inhibition (ZOI) against *E. coli:* (a) Control; (b) &(c) show zones of inhibition in different concentrations of mucus and ampicillin as positive control.

control against pathogenic bacteria, Ampicillin was utilized at a concentration of 0.5 mg per 1 mL of sterilized distilled water. The bacterial cultures were maintained at 37°C for a period of 24 hr. Following incubation, the diameter of the inhibition zones surrounding the wells was determined using a ruler, with measurements recorded in millimeters (mm).

Statistical analysis

Standard statistical tools like Mean, Standard Error and Student's *t* test were conducted to analyze data.

RESULTS AND DISCUSSION

Biochemical analysis of epidermal mucus

Protein was identified as a primary constituent in the skin mucus of this fish species. Our results showed that the concentration of total protein in mucus is 124±4.583 µg/mL. Carbohydrate is also an important component found in the epidermal mucus of *M. cuchia*. Our results shows that the total carbohydrate in mucus is 331±5.099 µg/mL.

An analysis of free fatty acids revealed that the skin mucus of *M. cuchia* contains a measurable quantity of lipids. The presence of lipids was verified by observing a color change from deep pink to light pink upon the addition of a standardized alkaline solution. Our results show that the free fatty acids content in mucus is 0.027±0.003 % or 0.001±0.0002 g in 5 g of mucus sample.

An analysis of free fatty acids revealed that the epidermal mucus of this fish species contains the smallest amount of lipids in comparison to its protein and carbohydrate content.

From the Table 1, our analysis revealed that the epidermal mucus of *M. cuchia* contains a higher proportion of carbohydrates compared to proteins and lipids. Among these components, lipids were found to be present in the smallest quantity within the epidermal mucus of this fish species, with proteins and carbohydrates being more abundant. The protein content in the epidermal mucus is 124±4.583 µg/mL, carbohydrate content is 331±5.099 µg/mL whereas the lipids or free fatty acid content is 0.027±0.003% of sample.

Table 1: Estimation of chemical components present in Epidermal mucus.

Components	Quantity
Total Proteins	124±4.583 µg/mL
Total Carbohydrates	331±5.099 µg/mL
Free fatty acids	0.027±0.003%

N.B. Data analysis has been done Mean±SEM, where n=5.

Table 2: Zones of Inhibition (ZOI) against two bacteria in different concentrations of epidermal mucus.

Zones of Inhibition (mm) [Expressed as Mean±SEM]						
	Different concentrations of epidermal mucus					Positive control
Bacterial strains	100%	60%	40%	30%	10%	Ampicillin (0.5 mg/mL)
E. coli	9.02±0.30	6.84±0.23	4.01±0.26*	3.69±0.23	1.47±0.22*	14.02±0.21
S. aureus	4.48±0.22	3.68±0.19	2.31±0.18*	1.81±0.15	0.71±0.02*	5.82±0.18

N.B. Mean±SEM, where n=5; * indicates $p \leq 0.05$.

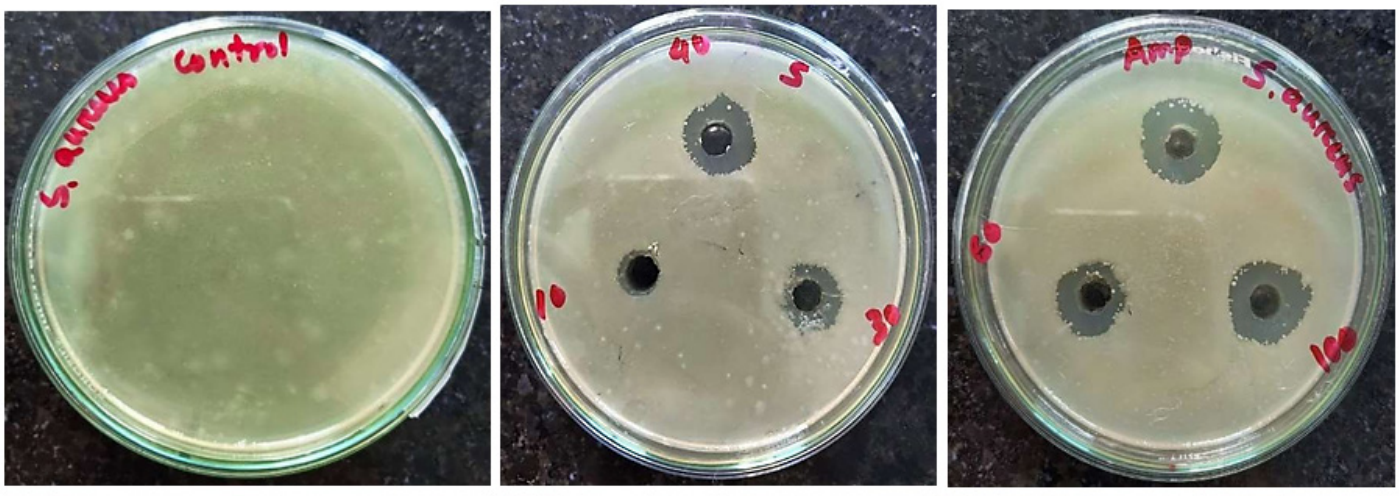

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Figure 3: Showing Zones of Inhibition (ZOI) against *S. aureus:* (a) Control; (b) and (c) show zones of inhibition in different concentrations of mucus and ampicillin as positive control.

Antimicrobial activity test of epidermal mucus

The antimicrobial activity of the skin mucus of *M. cuchia* was observed against two different bacterial stains, *Escherichia coli* and *Staphylococcus aureus*. The Zones of Inhibition (ZOI) were observed against two bacteria in different concentrations of epidermal mucus.

From Table 2, we observed that different concentrations of epidermal mucus show different zones of inhibition against the different bacteria. *M. cuchia* mucus was prepared in various concentrations of 100, 60, 40, 30 and 10% which show zone of inhibition of 9.02±0.30 mm,6.84±0.23 mm, 4.01±0.26 mm, 3.69±0.23 mm and 1.47±0.22 mm respectively against the *Escherichia coli* strain (Gram-negative bacteria). Like that the epidermal mucus of concentrations of 100, 60, 40, 30 and 10% which show zone of inhibition of 4.48±0.22 mm, 3.68±0.19 mm, 2.31±0.18 mm, 1.81±0.15 mm and 0.71±0.02 mm respectively against the *Staphylococcus aureus* strain (Gram-positive bacteria). The Ampicillin is used as a positive control that shows their activity against different stains. The zones of inhibition 14.02±0.21 mm and 5.82±0.18 mm is observed for ampicillin against *E. coli* and *S. aureus* strains respectively (Table). Figures 2 and 3 represents the comparison between the result showed by different concentration of epidermal mucus of *M. cuchia* against the two bacterial strains. The Zone of inhibition is highest in 100% concentration of epidermal mucus for both bacteria and it also showed neck to neck result with the positive control group. Tests of significance (t-test) have been done for all data analyses.

Research conducted by Elavarasi *et al.*, examined the proteins with bactericidal properties found in the skin mucus of freshwater fish species, including *Clarias batrachus* and *Tilapia mossambicus*.[8] This experiment revealed that the protein concentrations in skin mucus of *C. batrachus* was about 22 µg/mL, whereas this of *T. mossambicus* was about 34 µg/mL. In a comparable study, Anbuchezhian *et al.*, investigated the presence of antimicrobial peptides in the epidermal mucus of select Estuarine catfish species., such as *Aulostomus maculatus* and *Mystus gulio*.[9] This research revealed that *A. maculatus* had 11% of its total body protein in its mucus, while mucus of *M. gulio* contained 9% of its overall body protein. In the present study, the result obtained from epidermal mucus of *M. cuchia* showed that the concentration of total protein in mucus is 124±4.583 µg/mL as given in Table . A biochemical analysis of mucus extracts conducted by Ali *et al.*, 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*. This study also found that carbohydrate and lipid concentrations followed a similar pattern, with lower levels in the latter two species.[10] In the present study, the result obtained from epidermal mucus of *M. cuchia* indicated that the concentration of total carbohydrates in mucus is higher as compared to protein and lipids. The protein content in the epidermal mucus is 124±4.583 µg/mL, carbohydrate content is 331±5.099 µg/mL, whereas the lipids or free fatty acid content is 0.027±0.003% of sample as given in the Table . The result showed the zones of inhibition were 9.02±0.30 mm and 4.48±0.22 mm against the *E. coli* and *S. aureus* respectively when exposed to 100% of epidermal mucus of *M. cuchia*. The zones of inhibition were also higher in case of *E. coli* in all concentrations of epidermal mucus including the positive control ampicillin. This may be due to variations in bacterial cell structures among the bacteria.

CONCLUSION

The study finally revealed that skin mucus of *M. cuchia* have a very potential effect on two different bacterial strain i.e. *S. aureus* and *E. coli* and from this we can conclude that the skin mucus may help the organism to protect its skin from many soils bacterium when it is on its natural habitat i.e., mud. The biochemical analysis shows a very good presence of protein content, and which may indicate the presence of many antimicrobial peptides in the epidermal mucous of *M. cuchia*.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

ABBREVIATIONS

AMP: Antimicrobial Peptide; **BSA:** Bovine Serum Albumin; **MHA:** Mueller-Hinton agar; **OD:** Optical Density; **SEM:** Standard error of mean; **ZOI:** Zone of Inhibition.

SUMMARY

The freshwater mud eel *Monopterus cuchia*, native to North-East India, especially Assam, inhabits shallow, vegetation-rich environments like ponds, rivers, and beels, often in mud holes with low oxygen levels. To protect itself from soil bacteria, this species produces epidermal mucus. This study investigates the biochemical composition and antimicrobial properties of *M. cuchia*'s epidermal mucus. Biochemical analysis revealed high concentrations of carbohydrates, followed by proteins and lipids, with lipids being the least abundant. The antimicrobial activity, tested at concentrations of 100%, 60%, 40%, 30%, and 10% showed that the mucus exhibited significant inhibition against *Escherichia coli* and *Staphylococcus aureus*, with the highest activity observed at 100% mucus concentration, comparable to ampicillin. These findings suggest that the epidermal mucus of *M. cuchia* may contain novel antimicrobial agents, which could be useful in both fish and human health applications.

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