Metabolite Profiling of Epidermal Mucus of Freshwater Fish Species by HR-LCMS Analysis

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Abstract

Fish epidermal mucus is the first line of defense against environmental stressors and pathogens, which plays an important role in innate immune defense, osmoregulation and chemical communication in aquatic organisms. It contains a wide range of bioactive molecules that help in maintaining the mucosal homeostasis and mediate protection against pathogens. Despite its physiological significance, the small-molecule metabolite composition has yet to be fully investigated. The present study aimed to identify metabolites present in the epidermal mucus of four economically important freshwater fish species *L. rohita*, *H. fossilis*, *C. striata* and *O. niloticus* using High-Resolution Liquid Chromatography–Mass Spectrometry (HR-LCMS) in both positive and negative electrospray ionization modes. A total of 281 metabolites, belonging to diverse chemical classes including amino acids, short peptides, fatty acids and their derivatives, alkaloids, terpenoids, phenolics, were tentatively identified. Of these, 198 were found in positive ESI mode and 83 in negative ESI mode. Among these, 43 metabolites were shared by all four species, suggesting a potentially conserved mucosal metabolite profile. In fact, a few identified metabolites have been reported to have antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory and anticancer properties. Some metabolite differences were also found between species, probably due to their ecological niches, feeding habits and physiology. The results demonstrate the great chemical diversity of fish epidermal mucus and its possible use as a source for bioactive compounds. This study provides insights into fish mucosal biology and supports the exploration of fish mucus metabolites for aquaculture applications, fish health monitoring, and future pharmaceutical development.

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Introduction

According to the Food and Agriculture Organization of the United Nations, the growing population of the world, standard of living and globalization have overstretched the aquaculture industry, which has to increase food production by half of the century (FAO, 2016). The health and well-being of the aquatic animals is closely linked to the economic success of the modern aquaculture (Esteban *et al.*, 2012). In this context, Fish skin mucus is a versatile biological interface that is vital in defending fish against environmental stress, pathogenic microorganisms and physical injury. This mucosal layer serves as the primary line of innate immune defense which provides many immunologically-related factors, including esterases, immunoglobulins, complement factors, C-reactive proteins, phosphatases, lysozymes, Lymphocytes, proteolytic enzymes and lectins (Brinchmann *et al*., 2016; Bruce *et al.,* 2017). All these components play a part in host defense, lubrication, osmoregulation, and communication in aquatic organisms (Esteban, 2012; Dash *et al*., 2018). Moreover, fish skin mucus was also claimed to have an antibacterial effect in a number of species which was observed more effective on gram-positive than gram-negative bacteria, although bioactive components that are of interest remain unidentified (Haniffa *et al*., 2014; Guardiola *et al*., 2017). Accordingly, fish epidermal mucus is increasingly recognized as a promising yet underexplored source of numerous bioactive molecules. Therefore, mucus metabolomics is worth studying to identify the bioactive compounds and effective biomarkers related to fish health status. Such studies are particularly important because the composition and rheological properties of mucus are crucial for sustaining its various functions (Lai *et al*., 2009). Furthermore, mucus surfaces serve as important biological matrices whose composition varies among species and is influenced by various exogenous (stress, water temperature, pH and infections) and endogenous (sex and stage of development) factors (Esteban, 2012). Therefore, in order to provide accurate interspecific comparisons, standardized mucus collection and processing methods are essential.

Consequently, comparative metabolomic profiling of fish mucus can provide valuable insights into species-specific biochemical adaptations and defence mechanisms. *Labeo rohita*, *Heteropneustes fossilis, Channa striata* and *Oreochromis niloticus* are freshwater fish species that are widespread and highly adaptable to a variety of ecological niches. These species are different in their feeding, condition of the habitat and physiological reactions, which are likely to affect the biochemical structure of their mucus. Moreover, changes in mucus metabolite profiles may also be influenced by environmental elements, including temperature and seasonality. Such interspecies variation is crucial in research to comprehend adaptations of functionality and determine exclusive bioactive compounds. In this context, fish mucus has gained considerable research attention as a promising source of biologically active molecules that have potential pharmaceutical uses whose collection process can be simple and non-invasive, thereby allowing the real time analysis and timely tracking of molecules of interest, giving relevant information about the health status of the fish species (Ekman *et al*., 2015, 2023). With the advancement in the development of analytical technologies, metabolomics has become a powerful method for the overall characterization of complex biological systems. Of these methods, high-resolution liquid chromatography-mass spectrometry (HR-LCMS) has emerged as a pillar in metabolite profiling, and it provides high sensitivity, resolution, and accuracy. This method is especially appropriate in untargeted metabolomics research and helps in the identification of new compounds and metabolic processes (Ivanova *et al*., 2018). Despite of an increasing interest in fish mucus research, the majority of past studies mainly targeted the protein and peptide components, whereas comprehensive profiling of small-molecule metabolites remains comparatively limited. Thus, the present study aims to perform a detailed HR-LCMS-based metabolomic profiling of crude mucus obtained from four freshwater fish species, namely, *Labeo rohita*, *Heteropneustes fossilis, Channa striata* and *Oreochromis niloticus* to characterize their metabolite composition and identify potential species-specific bioactive compounds.

Materials And Methods

Experimental fish species and maintenance

Each of the four experimental fish species, *Labeo rohita*, *Heteropneustes fossilis*, *Channa striata*, and *Oreochromis niloticus* (150-200 g) were collected from local fish farms in Kurukshetra and acclimatized for 10-15 days before collecting mucus samples. These fish species were selected to represent a variety of freshwater fish species with distinct physiological adaptations. *L. rohita* is an important Indian major carp, *H. fossilis* and *C. striata* are air-breathing fish adapted to hypoxic environments, while *O. niloticus* is a highly adaptive and extensively cultivated species. The selected species facilitated a comparative evaluation of mucus metabolites among fishes with distinct ecological and physiological traits. Fish were kept in controlled environments in 600-litre fibreglass-reinforced plastic (FRP) aquariums and fed a formulated diet (40% protein) at 4% of body weight daily. Water quality was maintained by replacing 50% of the tank water regularly. Following a week of acclimatization, the fish were utilized for mucus collection. Only healthy fish were selected for further analysis.

Mucus sampling procedure

After acclimatization, Epidermal mucus was collected from the dorsal surface of the fish using sterile spatula by gently scraping in an antero-posterior direction (head to tail). Samples were obtained from five individuals of each

species. The ventral region was avoided to prevent contamination from intestinal and urinogenital sources (Chong *et al*., 2005). This treatment was done without the use of any anaesthesia and the initial mucus layer was discarded to reduce microbial contamination. Collected Samples were transferred into labelled 50 millilitre vials and immediately stored at 0°C to prevent bacterial growth and protein degradation.

Mucus extract preparation

For Crude mucus extract preparation, the collected epidermal mucus was centrifuged at 5000 rpm for 12 minutes. The resulting supernatant was separated and stored at 4°C for further analysis.

HR-LCMS Analysis

Crude mucus extract of experimental fish species was analysed at SAIF (Sophisticated Analytical Instrument Facilities) IIT Bombay, using HR-LCMS. The analysis was performed on an Agilent G6550A iFunnel Q-TOFs mass spectrometer equipped with a dual AJS Electrospray Ionization (ESI) source. The system was operated within a pressure range of 0.00-1200.00 bar, with a mass scan range of 120-1200 m/z. Chromatographic separation was achieved using a Hypersil GOLD C18 Column (100 mm × 2.1 mm × 3 micron particle size).

Operating conditions were as follows:

Gas temperature: 250°C; Gas flow: 13 L/min; Sheath gas temperature: 300°C; Sheath gas flow: 11 L/min; Nebulizer pressure: 35 psig.

Scan source Parameters:

Skimmer: 65 V; Capillary voltage (VCap): 3500 V; Fragmentor voltage: 175 V; Nozzle voltage: 1000 V; Octopole RF peak: 750.

Sampling handling parameters:

Draw position offset: 0.0 mm; Draw speed: 100 µL/min; Eject speed: 100 µL/min; Sample flush out factor: 5; Vial bottom sensing: enabled; post-draw wait time: 2 s.

Results

Epidermal mucus of freshwater fish species, *Labeo rohita*, *Heteropneustes fossilis*, *Channa striata* and *Oreochromis niloticus* were analysed to ascertain bioactive compounds with the help of HR-LCMS, along with their detailed mass spectra, mass-to-charge ratios, absorbance spectra and retention time for each. The total ion chromatograms obtained under positive and negative ESI modes are presented in Figures 1 and 2, respectively. The chromatographic profiles revealed numerous peaks distributed across different retention times, indicating the presence of diverse metabolites in the epidermal mucus of all four fish species. Following sample normalisation, variation in peak number, intensity and retention time among species suggests differences in mucus metabolite composition. A total of 198 and 83 bioactive compounds belonging to different classes, including amino acids, fatty acids and their derivatives, small peptides, etc. were tentatively identified in epidermal mucus of all experimental fish species via positive and negative HR-LCMS electrospray ionization respectively. In *L. rohita*, *H. fossilis*, *C. striata* and *O. niloticus*, a total of 51, 47, 51 and 49 compounds were identified using positive electrospray ionization and the remaining 22, 21, 08 and 32 compounds were identified using negative electrospray ionization respectively. Out of 281 compounds, 43 compounds were found to be common among all experimental fish species. Some of the commonly detected compounds includes O-Geranylvanillin, N-Carbamoylputrescine, 1-Pyrenylsulfate, 2,4-diamino-butyric acid, Lysyl-Glycine, 2-oxophytanic acid, (Z)-2-Hexenal, 1,2-Diacetylhydrazine, Ethambutol, 1H-Imidazole-4-methanol, Methylisocitric acid, Hexazinone, 2-Dodecylbenzenesulfonic acid, 2R-hydroxy-stearic acid, Hydrocortisone cypionate, Carphenazine, Promazine, Metharbital, S-Japonin, cis-Zeatin, Vanillin, etc. The results provide a glimpse into the complexity of the mucus matrix with chromatograms representing several compounds. The list of compounds identified in the HR-LCMS analysis of the epidermal mucus in different freshwater fish species is presented in Table 1, 2, 3 and 4.

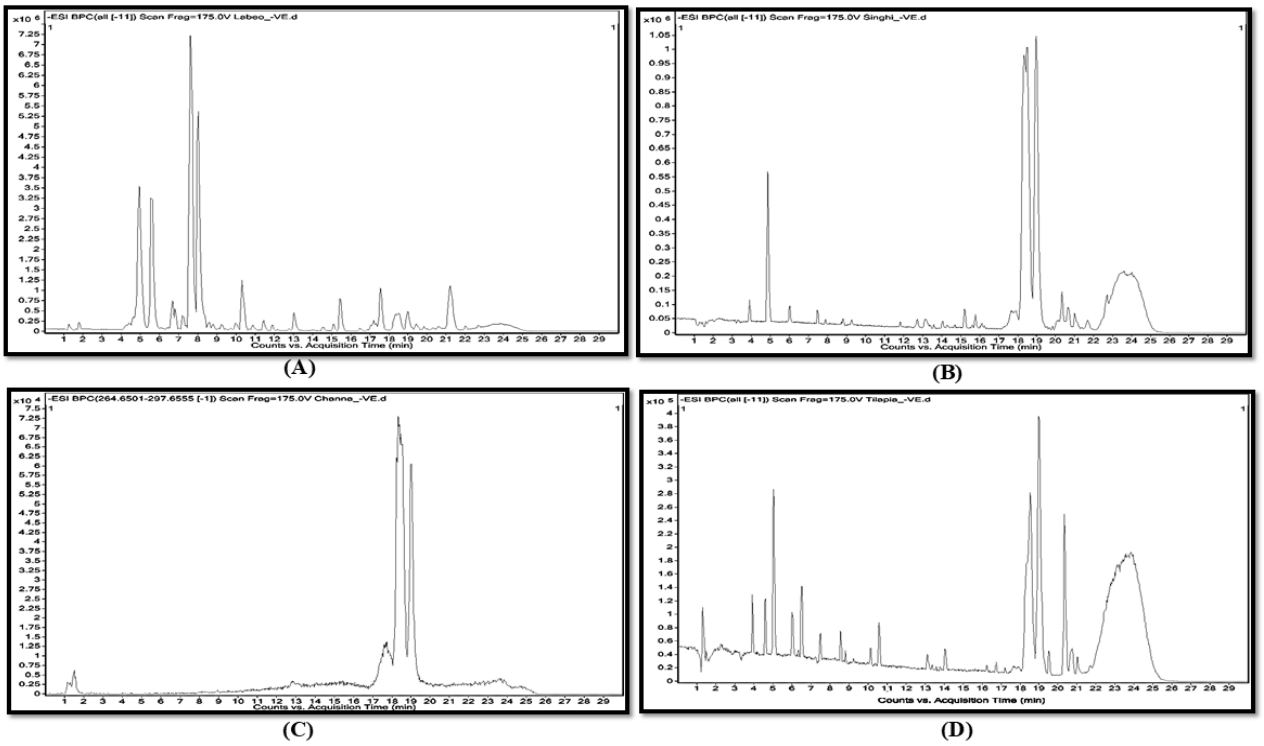

Figure 1: Representative total ion chromatograms (TICs) of epidermal mucus extracts from (A) *L. rohita* (B) *H. fossilis* (C) *C. striata* (D) *O. niloticus* obtained in positive electrospray ionization (ESI+) mode using HR-LCMS analysis

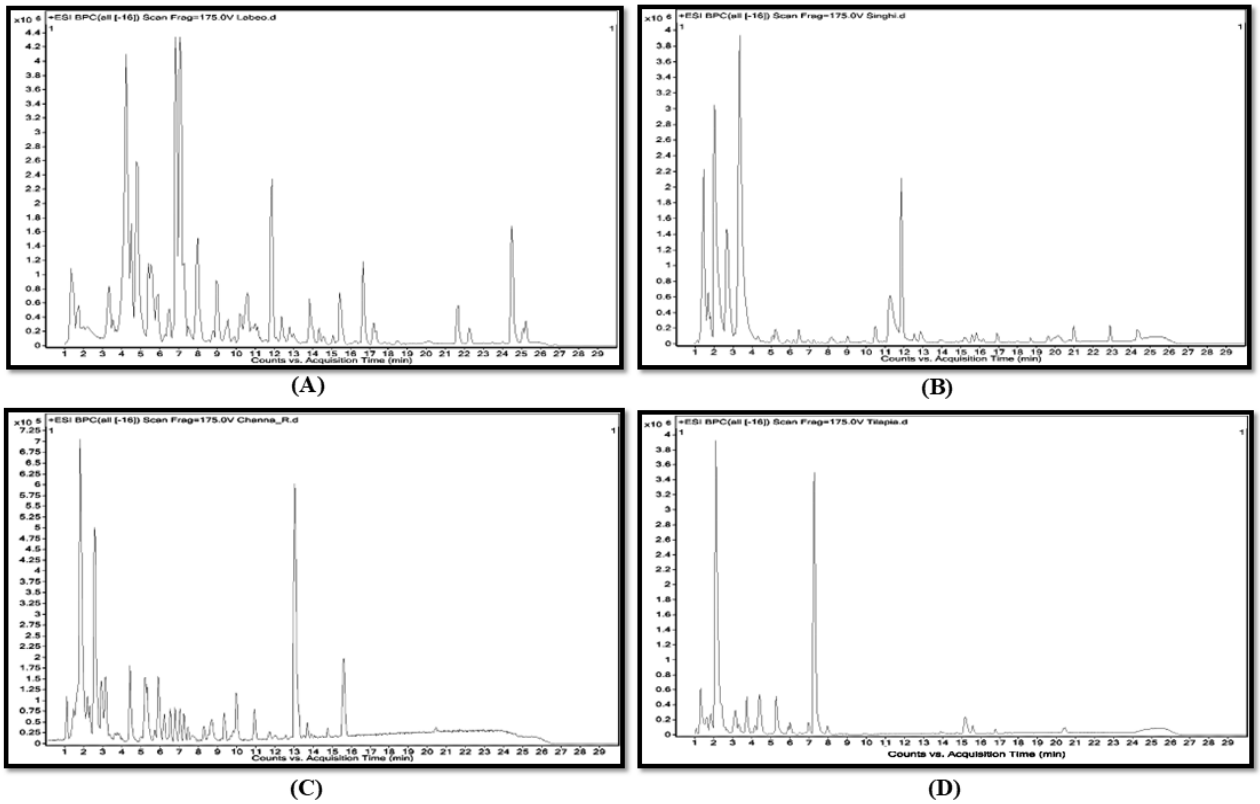

Figure 2: Representative total ion chromatograms (TICs) of epidermal mucus extracts from (A) *L. rohita* (B) *H. fossilis* (C) *C. striata* (D) *O. niloticus* obtained in negative electrospray ionization (ESI-) mode using HR-LCMS analysis

Table 1: Tentatively identified bioactive metabolites from epidermal mucus of *L. rohita* by HR-LCMS analysis

Bioactive Compounds	**Formula**	**Mass**	**M/z**	**Retention time (RT) (Min)**	**Reported biological relevance (literature-based)**	**References**
Acetyl tyrosine ethyl ester	$C_{13}H_{17}NO_4$	251.112	252.1193	1.417	Antibacterial and antioxidant	Ding *et al*., 2019; Sugumaran and Robinson, 2010
N-Carbamoylputrescine	$C_5H_{13}N_3O$	131.1059	154.0951	1.77	Regulate osmoregulation and stress response	Masson *et al*., 2017
2-Hydroxymethylserine	$C_4H_9NO_4$	135.0527	136.06	1.851	Antitumor activity	Ajaykumar *et al*., 2023
Ketotifen	$C_{19}H_{19}NOS$	309.1165	310.1236	4.406	Anti-inflammatory	Aldossary *et al*., 2026
Melithiazol A	$C_{20}H_{26}N_2O_4S_2$	422.1348	445.1239	4.602	Antifungal	Böhlendorf *et al*., 1999
Estramustine	$C_{23}H_{31}Cl_2NO_3$	439.1623	440.1694	4.666	Antiproliferative and Antimicrotubule activities	Piepmeier *et al*., 1993;
2,6-Dimethoxy-4-methylphenol	$C_9H_{12}O_3$	168.0779	191.0672	5.491	Anti-inflammatory and antibacterial properties	Iriawati *et al*., 2024
Cephradine	$C_{16}H_{19}N_3O_4S$	349.1096	350.1167	6.467	Antibacterial activity	Miraglia *et al*., 1973
Caulerpin	$C_{24}H_{18}N_2O_4$	398.1295	399.1371	12.423	Fungistatic activity	Nursidika *et al*., 2025
N-heptanoyl-homoserine lactone	$C_{11}H_{19}NO_3$	213.1347	236.124	9.609	Antibacterial activity	Dobretsov *et al*., 2011
Dipiperamide A	$C_{34}H_{38}N_2O_6$	570.2766	593.2658	24.466	Antifungal activity	Das *et al*., 2020.
N-Methyl-2,3,7,8-tetramethoxy-5,6-dihydrobenzophenathridine-6-ethanoic acid	$C_{24}H_{25}NO_6$	423.1664	424.1739	5.534	Antibacterial activity	Peng *et al*., 2023; Yang *et al*., 2024
Clavamycin F	$C_{15}H_{24}N_4O_7$	372.1633	395.1526	8.231	Antifungal and antibacterial activity	Finlay, 2003; Pruess and Kellett, 1983
Deoxypumiloside	$C_{26}H_{28}N_2O_8$	496.1875	519.1768	9.074	Antitumor and antiviral activity	Omer *et al*., 2021
Thalicpureine	$C_{22}H_{27}NO_5$	385.1893	408.1785	7.149	Antitumor and antimicrobial	Wang *et al*., 2024
Didanosine	$C_{10}H_{12}N_4O_3$	236.0909	237.0982	11.169	Antiviral activity	Rabie, 2022
cis-Piceid	$C_{20}H_{22}O_8$	390.1267	413.1154	11.319	Antioxidant and Antiproliferative	Su *et al*., 2013
2-Hydroxy-4-(4-methoxyphenyl)-1H-phenalen-1-one	$C_{20}H_{14}O_3$	302.0968	325.0861	7.501	Antifungal and Antimicrobial	Otálvaro *et al*., 2007; Song *et al*., 2017

Table 2: Tentatively identified bioactive metabolites from epidermal mucus of *H. fossilis* by HR-LCMS analysis

Bioactive Compounds	Formula	Mass	M/z	Retention time (RT) (Min)	Reported biological relevance (literature-based)	References
Pseudoisoeugenol 2-methylbutanoate	$C_{15}H_{20}O_3$	248.1446	249.1519	1.348	Antibacterial and antioxidant	da Silva *et al*., 2018
1H-Imidazole-4-methanol	$C_4H_6N_2O$	98.0486	121.0378	1.851	Antifungal	Borgers *et al*., 1980
Avenanthramide 1c	$C_{16}H_{13}NO_5$	299.0824	322.0716	1.863	Anti-inflammmatory	Sasaki *et al*., 2026
(Z)-2-Hexenal	$C_6H_{10}O$	98.0738	121.063	2.217	Antibacterial activity	Nakamura and Hatanaka, 2002
Tris(1-aziridinyl)phosphine oxide	$C_6H_{12}N_3OP$	173.0719	196.0611	3.106	Anticancerous activity	Bburgos-Moron *et al*., 2021
Clenbuterol	$C_{12}H_{18}Cl_2N_2O$	276.0762	277.0842	3.131	Anti-tumor	Henschel *et al*., 2025
Amodiaquine	$C_{20}H_{22}ClN_3O$	355.1445	356.1521	3.141	Antiviral activity	Sakurai *et al*., 2018
3'-Glucosyl-2',4',6'-trihydroxyacetophenone	$C_{14}H_{18}O_9$	330.0939	331.1037	3.211	Antibacterial activity	Olatunde *et al*., 2021
2-Hexylbenzothiazole	$C_{13}H_{17}NS$	219.1071	220.1143	3.779	Antibacterial activity	Sabira *et al*., 2025
Ethambutol	$C_{10}H_{24}N_2O_2$	204.1826	227.1718	4.338	Antibacterial activity	Liss *et al*., 1982
Fospropofol	$C_{13}H_{21}O_5P$	288.1134	289.1206	5.069	Antioxidant	Sabira *et al*., 2025
9-(3,4-Dimethoxyphenyl)-2-methoxy-1H-phenalen-1-one	$C_{22}H_{18}O_4$	346.121	347.1281	5.811	Antifungal activity	Quinones *et al*., 2000
Spiromesifen	$C_{23}H_{30}O_4$	370.213	393.2024	5.865	Antiviral activity	Shady *et al*., 2024
Sulfadoxine	$C_{12}H_{14}N_4O_4S$	310.0747	311.082	10.778	Antiparasitic	Mlugu *et al*., 2020
Amastatin	$C_{21}H_{38}N_4O_8$	474.2692	497.2585	11.707	Antifungal activity	Aoyagi *et al*., 1978

Table 3: Tentatively identified bioactive metabolites from epidermal mucus of *C. striata* by HR-LCMS analysis

Bioactive Compounds	Formula	Mass	M/z	Retention time (RT) (Min)	Reported biological relevance (literature-based)	References
Promazine	$C_{17}H_{20}N_2S$	284.1329	285.1401	3.079	Antibacterial activity	Ronco *et al*., 2020
Carphenazine	$C_{23}H_{29}N^3O_2S$	411.1921	434.1816	2.428	Antibacterial activity	Nehme *et al*., 2018
Lucidenic acid A	$C_{27}H_{38}O_6$	458.2641	481.2534	6.505	Antimicrobial, antiviral and anticancerous activity	Zheng *et al*., 2023
Saponins	$C_{19}H_{28}O_3S$	336.1733	337.1806	4.455	Antibacterial activity	Francis *et al*., 2002
D-Linalool 3-(6" malonylglucoside)	$C_{19}H_{30}O_9$	402.1864	403.1937	13.491	Antimicrobial	Zhong *et al*., 2021

Pentoxifylline	$C_{13}H_{18}N_4O_3$	278.1375	279.1449	13.274	Anti-inflammatory	Groesdonk *et al*., 2010
Flurandrenolide	$C_{24}H_{33}FO_6$	436.2197	437.2271	6.197	Anti-inflammatory	Boris *et al*., 1977
Xanomeline	$C_{14}H_{23}N_3OS$	281.1586	282.165	9.612	Inhibit oxidative stress and Apoptosis	Xin *et al*., 2020
Enalaprilat	$C_{18}H_{24}N_2O_5$	348.1696	349.1766	5.481	vasoprotective	Prasad *et al*., 1999

Table 4: Tentatively identified bioactive metabolites from epidermal mucus of *O. niloticus* by HR-LCMS analysis.

Bioactive Compounds	**Formula**	**Mass**	**M/z**	**Retention time (RT) (Min)**	**Reported biological relevance (literature-based)**	**References**
N-Carbamoylputrescine	$C_5H_{13}N_3O$	131.1059	154.0951	1.558	Role in growth and stress tolerance	Masson *et al*., 2017
(Z)-2-Hexenal	$C_6H_{10}O$	98.0738	121.063	2.217	Antibacterial activity	Nakamura and Hatanaka, 2002
Actinamine	$C_8H_{18}N_2O_4$	206.1248	229.1145	1.711	Antibacterial activity	Scarff *et al*., 2019
N,N'-Diphenyl-p-phenylenediamine	$C_{18}H_{16}N_2$	260.1326	261.14	1.93	Antioxidant and Potential Anticancer	Ali *et al*., 2013; Elyoussoufi, 2012
1H-Imidazole-4-methanol	$C_4H_6N_2O$	98.0486	121.0377	1.844	Antimicrobial and Anticancerous	Al Ghamdi *et al*., 2024; Parmar *et al*., 2023
4-Chlorophenylacetic acid	$C_8H_7ClO_2$	170.0122	171.0197	2.328	Antifungal and Antibacterial activity	Hwang *et al*., 2001
Isoleucyl-Alanine	$C_9H_{18}N_2O_3$	202.1288	203.1363	1.572	Antimicrobial and antiparasitic properties	Álvarez *et al*., 2024
Lysyl-Glycine	$C_8H_{17}N_3O_3$	203.1258	226.1148	1.652	Antimicrobial and antiparasitic properties	Álvarez *et al*., 2024
Ricinoleic acid	$C_{18}H_{34}O_3$	298.2467	321.2357	14.862	Antimicrobial	Park *et al*., 2020
Ethambutol	$C_{10}H_{24}N_2O_2$	204.1823	227.1716	4.41	Antibacterial activity	Liss *et al*., 1982
Maculosin	$C_{14}H_{16}N_2O_3$	260.1192	261.1265	4.416	Antibacterial activity	Driche *et al*., 2022
2-Hexyl-4,5-dimethylthiazole	$C_{11}H_{19}NS$	197.125	220.1143	3.765	Antimicrobial and antiproliferative agents	Sharma *et al*., 2019
4-Chloroacetophenone	C_8H_7ClO	154.018	155.0252	2.448	Antimicrobial activity	Hussain *et al*., 2021

Hispidulin	$C_{16}H_{12}O_6$	300.0606	323.0503	5.26	Anticancer and antiviral activity	Patel and Patel, 2016
S-Japonin	$C_{19}H_{28}O_3S$	336.1742	337.1815	4.432	Antiproliferative activity	Arisawa *et al*., 1989
Methyl 7-epi-12-hydroxyjasmonate glucoside	$C_{19}H_{30}O_9$	402.1869	403.1941	13.483	Anticancerous and antiviral potential	He *et al*., 2019

Discusssion

Over the past few decades, natural products have emerged as an important focus of antimicrobial research due to their potential for the development of novel therapeutic agents. As naturally derived substances, they are often regarded as environmentally friendly and biologically safe alternatives to synthetic compounds (Adnan *et al*., 2017; 2018). Among the various natural sources investigated, fish mucus has gained increasing attention due to its rich composition of bioactive molecules and its crucial role in host defense. One such example is Hagfish, one of the most primitive vertebrates, which lacks several key components of adaptive immunity, including antibody-mediated immune responses and a thymus, that are commonly present in teleost fishes (Raison *et al*., 1998; Rolf *et al*., 2007). Despite these limitations, hagfish thrive in pathogen-rich muddy ocean habitats as scavengers (Spitzer *et al*., 1998). Their ability to survive under such conditions is thought to be associated with the secretion of large quantities of mucus containing antimicrobial peptides, lysozyme, proteases and other bioactive compounds that provide effective protection against microbial invasion (Subramanian *et al*., 2007). Therefore, in the search for natural antimicrobial and bioactive compounds, mucus extract from four economically important Freshwater fish species *L. rohita*, *H. fossilis*, *C. striata*, and *O. niloticus*, was investigated. The mucus extract of these potentially medicinally important fishes exhibited a broad spectrum of bioactive compounds, with HR-LCMS analysis tentatively identifying a total of 281 metabolites across the species, consistent with the recent metabolomic studies highlighting fish mucus as a rich reservoir of diverse metabolites with potential functional significance (Ekma *et al*., 2023)

The aquatic environment harbours a vast diversity of pathogenic and non-pathogenic microorganisms, and fish remain in constant interaction with this microbe-rich habitat. Consequently, the fish skin functions as a unique mucosal surface where living epidermal cells are continuously exposed to opportunistic bacteria, fungi, and viruses (Magnadottir, 2010). To cope with these environmental challenges, fish secrete mucus that serves as a crucial protective barrier. Many studies have highlighted fish mucus as a potent source of novel antimicrobial compounds, acting as the first line of defense against invading pathogens (Arellano *et al*, 2004; Mozumder, 2005) In the present study, HR-LCMS was used to characterize the fish "mucolome". In these species, a total of 281 metabolites were tentatively identified, belonging to diverse array of amino acids, peptides, fatty acid derivatives, alkaloids, terpenoids and phenolics (Ivanova *et al*., 2018). The antibacterial, antioxidant, anti-inflammatory, antiviral and anticancerous activities have been previously reported for several of these compounds. These findings illustrate the high chemical complexity of the fish mucosal barrier and its multifunctional biological relevance. Epidermal mucus is not only a physical lubricant, but also a dynamic, living interface that regulates the first line of innate immunity against aquatic pathogens (Shephard, 1993; Lee *et al*., 2020). These metabolites reported a wide range of biological activities, especially antimicrobial and antioxidant properties, which are necessary for mucosal immunity. Compounds such as Maculosin, Promazine, Ethambutol, Acetyl tyrosine ethyl ester, Pseudoisoeugenol 2-methylbutanoate, Saponins, D-Linalool 3-(6" malonylglucoside), and Lucidenic acid A have previously been reported for their antibacterial and antimicrobial activities (Liss *et al*., 1982; Kim *et al*., 1999; Francis *et al*., 2002; Sugumaran and Robinson, 2010; da Silva *et al*., 2018; Ding *et al*., 2019; Ronco *et al*., 2020; Zhong *et al*., 2021; Zheng *et al*., 2023). In particular, Promazine, a phenothiazine derivative, exhibits significant antibacterial activity against *Staphylococcus aureus*. Its antimicrobial effect has been associated with the disruption of bacterial cellular functions, suggesting a potential contribution to the antibacterial defense properties of fish mucus (Ronco *et al*., 2020).

On the other hand, studies have reported that free fatty acids are a significant component of piscine mucus and play an integral role in protection by inhibiting various cellular activities, such as disrupting bacterial membranes, inhibiting enzyme activity, uncoupling oxidative phosphorylation, disrupting the electron transport chain, and inducing cell lysis (Lewis *et al.*, 1970; Desbois *et al*., 2009). The detected fatty acids and their derivatives, such as Ricinoleic acid, Pseudoisoeugenol 2-methylbutanoate, have been documented to disrupt microbial cell membranes or inhibit essential metabolic pathways in pathogens (da Silva *et al*., 2018; Park *et al*., 2020). For instance, the presence of various amino acid derivatives like Acetyl tyrosine ethyl ester, a non-proteinogenic amino acid, contributes to the antioxidant capacity of the mucus (Sugumaran and Robinson, 2010; Ding *et al*., 2019), which may provide a surrogate measure of the localised proteolytic environment, which has been implicated in the activation of innate antimicrobial peptides (Ding *et al*., 2019; Díaz-Puertas *et al*., 2023). In addition, the anti-inflammatory metabolites such as Flurandrenolide, Pentoxifylline, Avenanthramide 1c identified in this study are essential for maintaining tissue integrity in fish continuously exposed to environmental stressors, toxins and physical abrasions. Such condition requires a rapid yet tightly regulated inflammatory response to prevent chronic tissue damage and maintain mucosal homeostasis (Boris *et al*., 1977; Groesdonk *et al*., 2010; Sasaki *et al*., 2026). Furthermore, the detection of compounds such as Ketotifen, Estramustine, Hispidulin, Lucidenic acid A, and Amodiaquine, which have been reported to exhibit antiproliferative and antiviral activities, suggests that fish mucus may represent a repository of bioactive molecules with diverse

physiological functions (Piepmeier *et al*., 1993; Patel and Patel, 2016; Sakurai *et al*., 2018; Zheng *et al*., 2023). In addition to antibacterial, antiviral, and anti-inflammatory compounds, the HR-LCMS analysis tentatively identified several compounds, such as Lucidenic acid A, Clauperin, Amastatin, 1H-Imidazole-4-methanol, Tris(1-aziridinyl)phosphine oxide, which have been reported in previous studies to exhibit antifungal and anticancer activity (Aoyagi *et al*., 1978; Borgers *et al*., 1980; Bburgos-Moron *et al*., 2021; Parmar *et al*., 2023; Zheng *et al*., 2023; Nursidika *et al*., 2025). For instance, Zheng *et al,* (2023) also reported that Lucidenic acid A exhibits significant anticancer potential by inducing cytotoxicity, promoting apoptosis, and suppressing proliferation and invasion in various cancer cell lines. In addition to these, Amastatin, an aminopeptidase inhibitor, has been reported in preliminary studies to exhibit antifungal properties (Aoyagi *et al*., 1978). The detection of this metabolite in fish mucus suggests its possible involvement in innate immune defense and antimicrobial protection, although further studies are required to confirm its significance in fish epidermal mucus. Along with these, 43 metabolites were found to be common across all four species, suggesting a conserved metabolic signature essential for teleost mucosal homeostasis (Ekman *et al*., 2023). Among these, N-Carbamoylputrescine, an intermediate in the polyamine biosynthetic pathway, plays a vital role in cell proliferation and stress tolerance (Masson *et al*., 2017). Polyamines are also known to modulate ion channels, an important physiological function for freshwater fish coping with osmoregulatory challenges (Guan *et al*., 2016). Likewise, the detection of Lysyl-Glycine, a bioactive dipeptide, may indicate the degradation of larger antimicrobial peptides or proteins or serve as a signalling molecule in the recruitment of immune cells (Álvarez *et al*., 2024). Interestingly, all the species contain (Z)-2-Hexenal. Its presence in fish mucus indicates either a nutritional origin or a function as a semiochemical involved in chemical communication or alarm signalling, despite the fact that it is frequently linked to plant "green leaf volatiles" (Reverter *et al*., 2018). The overall metabolite profiles were quite distinct among the four species, even though several conserved metabolites were present, suggesting ecological and physiological specialisation. In contrast to the predatory fish species, the herbivorous column feeder *L. rohita* showed a distinct metabolic emphasis. The feeding habits and habitat of predatory species such as *C. striata* and *H. fossilis*, an air-breathing bottom feeder, often expose it to different pathogenic pressures, requiring a more comprehensive or specialized suite of bioactive compounds (Ekman *et al*., 2023; Heim *et al*., 2023). Furthermore, the specificity of the presence of some alkaloids and terpenoids in *H. fossilis* might be a response to the benthic and often hypoxic environment with high microbial content (Graham *et al*., 1997; Saha *et al*., 2007). To facilitate the detailed characterization of these species-specific metabolite profiles, HR-LCMS was used, which allowed identifying almost 281 metabolites with high mass accuracy and sensitivity to a good degree of certainty (Ivanova *et al*., 2018, 2021). The HR-LCMS capability of detecting a broad spectrum of polar and non-polar metabolites, from simple amino acids to complex secondary metabolites such as Acetyl tyrosine ethyl ester, provides a powerful tool and versatility for aquatic metabolomics studies (Ivanova *et al*., 2018).

In addition to improving our knowledge of fish mucosal biology, such thorough metabolite profiling supports the increasing use of mucosal surfaces as a useful matrix for environmental surveillance and fish health evaluations, providing a less invasive method than blood or tissue sampling (Ekman *et al*., 2015, 2023). Apart from its ecological and diagnostic value, the richness of bioactive compounds found in fish mucus of *L. rohita*, *C. striata*, *H. fossilis* and *O. niloticus* underscores the potential of fish mucus as a promising reservoir for drug discovery (Diaz-Puertas *et al*., 2023). This potential is particularly significant considering the increasing threat of antibiotic resistance on a worldwide scale, which has led to an urgent need for novel antimicrobial medicines. Therefore, several metabolites found in this study might potentially serve as significant templates for the development of pharmaceuticals. Furthermore, metabolites with antioxidant and anti-inflammatory properties may have potential therapeutic applications in wound healing and dermatological treatments in human use (Hilles *et al*., 2019). However, further research is required to understand the biological activities, mechanisms of action, and therapeutic potential of these metabolites. It is crucial to conduct such investigations to enable metabolomic findings to be applied in the field of biomedicine and realise the therapeutic value of natural products from fish.

Conclusion

In the present study, HR-LCMS-based metabolomic analysis of the epidermal mucus from four freshwater fish species, namely *L. rohita*, *H. fosillis*, *C. striata* and *O. niloticus*, has been carried out in detail. There was a total of 281 metabolites that were tentatively identified, and include a wide variety of classes of bioactive compounds, such as amino acids and their derivatives, fatty acids and their derivatives, alkaloids, terpenoids, phenolics, etc., demonstrating the great biochemical complexity of fish mucus. The metabolites identified had a wide range of reported biological functions, such as antimicrobial, antioxidant, antiviral, anti-inflammatory, antifungal and anticancer effects, highlighting the multifunctional role of mucus in host defense and physiological regulation. The presence of 43 metabolites common to all four species suggests a potentially conserved metabolic framework linked to innate immunity and mucosal homeostasis, whereas species-specific differences in metabolite composition may reflect ecological adaptations and variable physiological requirements. The results indicate that the epidermal mucus of fish is a functional complex biological matrix but an unexplored source of bioactive natural compounds. Additionally, it can be used in a non-invasive manner, providing valuable information for fish health assessment and environmental monitoring. Together, all findings point to the possibility of fish mucus serving as a source of new molecules with therapeutic potential for pharmaceutical and biotechnological use. The isolation, structural confirmation and biological validation of metabolites will be essential to future studies to

better understand their mechanisms of action and to fully realise their therapeutic potential.

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Authors' contributions

AB conceived the idea, designed the experiment and participated in the preparation of the manuscript. PY collected the material, carried out the experiment and analyzed the samples/data. Both authors read and approved the final manuscript.

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