Impact of Seasonal Changes and Habitat on Biochemical and Antifungal Property of Fish Mucus

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Abstract

The present study was made to find the anti-microbial activity of the mucus collected from the,*Catlacatla, Labeo rohita* and *Cirrhinusmrigala*from the winter, summer and rainy seasons. The mucus collected were tested against three pathogenic fungus namely *Trichophyton rubrum , Fusarium soleni and Aspergillus niger* using Muller Hinton agar plates by using disc diffusion method and also compare quality of integumentary mucus according to season and water quality. Fish mucus having different unwilling parameter present which are immunoglobulin's, pathogen peptide and harmonize factors that provide both physical and mechanical protection. In the present study we find out, the seasonal effects of fish mucus on chemical composition, enzymatic activity and antifungal activity.

Key words: Fish skin mucus, antifungal activity, fishpathogen, seasonal effect

Introduction

Fish are a diverse group of animals, highly specialized for their aquatic existing and comprising almost half number of vertebrate species in existence today. They are in intimate contact with their environment, which can contain very high concentration of microbial organisms and different kinds of pathogens. Many of these are saprophytic, some are pathogenic and both are capable of digesting and degrading the fish tissue. The biological interface between fish and their aqueous environment consists of a mucus layer composed of biochemically diverse secretions from epidermal and epithelial cells (Ellis et al., 1999); (Ebran *et al.,* 2000);(Kuppulakshmi *et al*., 2008).Fish Integumentary mucus secreted by epidermal layer of the integument contains a slimy and slippery layer, that is mucin, made of water, antibacterial proteins and enzyme. It was reported that it contains different types of saturated fatty acid (SFA) monosaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA).and plays a key role in intra- and inter specific chemical communication (Leonard *et al*., 2012);(Todd *et al.,*1967);(Beklioglu *et al.,* 2006)

provide main surface of exchange between fish and their surrounding environment. Mucus also acts as a dynamic physical and biochemical barrier, displaying numerous biological and ecological roles such as osmoregulation (Shephard, 1993; Shephard, 1994), protection against abrasion (Oosten, 1957), protection against environmental toxins and heavy metal toxicity (Coello *et al*., 1996), parental feeding (Chong *et al*., 2006), protection against pathogens (Gomez *et al*., 2013).

Fish mucus research has increased in the last ten years mainly due to the discovery of numerous bioactive molecules (antibacterial, antiviral, antifungal, and antiparasitic) and their potential application in human medicine and in aquaculture (Rajanbabu*et al*., 2011; Rakers *et al*., 2013; Yano, 1996; Beck *et al.,* 2015; Shephard, 1994).

The skin mucus is mechanical protective in nature and it has an important component of the innate immune mechanism playing an important role in fish health (Subramanian *et al*., 2007, Depending on the fish species, skin mucus varies considerably in viscosity, thickness, and glycoprotein (mucin) content which also represent the major components of mucus (Dash *et al*., 2018),and also contains a variety of immune cells such as macrophages, lymphocytes, eosinophilic granulocytes, dendritic cells, organized cytokine response (Gomez *et al*., 2013). The antimicrobial property of crude epidermal mucus against infectious pathogens was initially demonstrated in rainbow trout (Austin & McIntosh 1988).

Material and methods

Collection of water

The water sample was collected from pond present in Dau Biharilal Fish Farm situated in Pulgaon area of Durg district. The latitude of study area is 21.167907 and longitude is 81.261727. The water sample was collected in three seasons, winter, summer and rainy season, in morning hours between 9 to 11 am in bottles. The water sample were immediately brought to laboratory for estimation of various physico-chemical parameters like water pH, Turbidity, Total Suspended Solids (TSS), Total Dissolved solids (TDS), Total Alkalinity (as $CaCO_3$), Total Hardness (as $CaCO_3$), Calcium (as Ca), Magnesium (as Mg), Dissolved Oxygen (as O_2), Chemical Oxygen Demand (C.O.D.), Biological Oxygen Demand (B.O.D.) free Residual Chlorine estimated in the laboratory by using Standard laboratory methods. Present study involves the Analysis of water quality in terms of physicochemical methods. (APHA 2005).

Fig 01:Sample site at Dau Biharilal fish farm Durg, C.G.

Selection of fishes: Three fishes species (*Catla catla,Labeo rohita* and *Cirrhinus mrigala*) from freshwater was collected directly from pond of Dau Bihari Lal fish farm Durg, Chhattisgarh which represent different feeding habits and live in different habitats (Surface dwellers, Mid dwellers and bottom dwellers) were selected for experiment. All three Species were identified following (Day1878and1889)**;**(Talwar and Jhingran1991); (Gopa kumar *et al*.,1999). Live fish specimen irrespective of sex, average range length of 18 centimetre(cm) to 30 cm and weighing 180-190 grams of each species were directly collected from Dau Bihari Lal fish farm Durg, Chhattisgarh.

Collection of mucus from fish: Mucus sample was collected from integument of Fish species (*Catla catla ,Labeo rohita* and *Cirrhinus mrigala*). by a modified method of (Subramanian *et al*., 2008). On the day of mucus collection fish was washed and transferred into a sterile polyethylene bag for 10 to 20 minutes and moved front and back to slough off the fish mucus (Wei *et al*., 2010). The mucus was immediately transferred to 15mL sterile centrifuge tubes and placed on dry ice. (Subramanian *et al*., 2008).

Protein estimation by Lowry method- (Lowry *et al*., 1951).

Carbohydrateestimation- Anthrone method- (Trevelyan *et al.,* 1952).

Enzyme estimation:

Protease Activity (PR): PR activity was determined by using azocasein hydrolysis assay, following the method described by (Subramanian *et al.,*2007) with slight modifications.

Esterase Activity (ES): ES activity assay was conducted following the method of (Palaksha *et al*., 2008). ES activity was determined continuously over 2-3 h at 405 nm using p-nitrophenyl myristate substrate (Sigma USA).

Lysozyme Activity (LY): The rate of LY activity was determined by turbidimetric method as described by (Subramanian *et al*., 2007).

Antifungal activity of mucus:

In vitro antimicrobial evaluation: Mucus samples were obtained from all three fishes *Catla catla, Labeo rohita, Cirhinnus mrigala* found in different habitat (surface dweller, mid dweller and bottom dweller) collected from fish farm. After the collection of mucus antifungal evaluation were carried out against three fungal strains *Fusarium soleni* (MTCC No.-350), *Aspergillus niger*(MTCC No.-282), *Trichophyton rubrum* (MTCC No.-8477). All the fungal strain obtained from the Microbial Type Culture Collection and Gene Bank (MTCC) CSIR-Institute of Microbial Technology, Sector-39A Chandigarh-160036, and India.

Statistical analysis: One way - analysis of variance (ANOVA) followed by Duncan's multiple range test (Duncan, 1955)for all the experiments was used to determine the significant variation between the different treatments. Student't' test was also used to determine the significant difference between different treatments. Statistical significance was settled at a probability value of $P<0.05$. All statistics were performed using SPSS Version 11.5 for Windows.

Result and discussion:

pH: Hydrogen ion concentration of water sample ranged from 6.87 ± 0.04 (winter) to 7.93 ± 0.03 (summer). Seasonally pH was recorded to be higher in summer season and lower in

winter season. The value of pH of rainy season was intermediate between summer and winter season which is 7.51±0.035.

Turbidity: The water is found to be most turbid in rainy season with the value 297 ±0.5 and least turbid in summer season 72.0±0.5. The turbidity of sample water in winter season is 198.8±0.5.

Total Suspended Solids (TSS): Minimum value of total suspended solid in water sample is found to be 176.6±0.05 mg/l in summer season followed by 260.0±1 mg/l in winter season and 262±1 mg/l in rainy season.

Total Dissolved Solids (TDS): TDS is found to be maximum in summer season followed by rainy season and minimum in winter season. Total dissolved solids in the pond water sample in all the three seasons were recorded to be 478.0±0.5 mg/l in winter, 618.0±1 mg/l in summer and 488±1.5 mg/l in rainy season.

Total Alkalinity (as $CaCO_3$) and Total Hardness (as $CaCO_3$): Seasonal alkalinity and hardness of water was recorded higher in summer season and lowest in rainy season. The value of winter season was intermediate for both alkalinity and hardness.

Calcium (as Ca) and Magnesium (as Mg): Calcium and Magnesium concentration was recorded highest in summer season i.e. 99.40±1.5 mg/l and 36.94±0.5 mg/l respectively.

Acidity: Acidity of the water sample was found to be maximum in summer season with a value 35.0±0.5 mg/l and minimum in rainy season having a value of 1.0±0.15 mg/l. Winter season shows intermediate value of acidity i.e. 12.0±0.2 mg/l.

Dissolved Oxygen (as O_2) and Biochemical Oxygen Demand (B.O.D.): Dissolved oxygen and Biological oxygen demand are recorded maximum in rainy season with a value 7.7±0.03 mg/l and 74.0±0.5 mg/l respectively and Do and BOD is recorded minimum in winter season having a value 4.3±0.05 mg/l and 23.33±0.1 mg/l respectively.

Chemical Oxygen Demand (C.O.D.): Chemical oxygen demand is maximum in summer season 113.68±1.5 mg/l and minimum in winter 88.0±1 mg/l.

Characteristics of skin mucus of fish : Fish possess numerous distinct and complex defence mechanisms to protect themselves from these pathogenic infections amongst which fish skin mucus acts as the first line of physical defence against pathogens (Wang *et al.,* 2011), which provides a stable physical or chemical barrier against the invading pathogens. It contains many important elements such as antimicrobial factors, proteins, lysozyme, immunoglobulin, and lectins (Dash *et al*., 2018), antimicrobial (AMPs) (Salinas, 2015).

Observations of skin mucus of fishes during mucus collection: Live specimen of length 19-34 cm and weight 190 - 910 grams of each specieswere selected for collection of mucus. Mucus was scrapped off at regularintervalpermonth. Mucus secretion when compared on habitat basis (mid, surface and bottom dwellers) During the study when we compare mucus secretion according to their habits, It was also observed that secretion of mucus was higher in surface dweller fish *Catla catla* compared to *Labeo rohita* and *Cirrhinus mrigala and* selected fishes secreted more mucus during summer season as compared to rainy and winters. It was also observed that during breeding season mucus secretion was high and more viscous.

Table No. 01: Average weight, average length and appearance of mucus of experimental fishes

Fish	**Average length (Cm)**	**Average weight (G)**	General observation of mucus
Catla catla	**21–30**	**300–350**	**Large amount of mucus and viscous in nature.**
Labeo rohita	**21 –32**	**300–350**	**Secreted moderate amount of mucus and quit clear mucus.**
Cirrhinus mrigala	**21 –32**	**300–350**	**Very low quantity and watery mucus.**

Table No 02: Amount of mucus extracted from healthy experimental fishes (in ml)

Fishes	**Amount of mucus(ml) per season / sample**		
Season	**Winter**	**Summer**	**Rainy**
Catla catla	4 – 6	5-8	3-4
Labeo rohita	3- 4	4-5	2-3
Cirrhinus mrigala	2-4	3-5	2-3

Biochemical composition of skin mucus: The mucosa consists of the mucous membrane and its underlying connective tissue and humoral part consists of the extracellular molecules present in the skin mucus (Salinas *et al.,* 2011). The skin mucosa of fish has different components such as protein, carbohydrates, lipids metabolites (Zaccone *et al.*,2001) and an essential barrier and serves as a protection against the surrounding environment with biotic and abiotic factors. The components present in mucus are-Water-65-90%,Proteins-10-22%,Fats–1-20%.

Characteristics of skin mucus of fish: Biochemical composition, enzymatic activity ofskin mucus of three fresh water Indian major carps *Cirrhinus mrigala, Catla catla* and *Labeo rohita*was carried out and also study the antifungal potency against three bacterial *strains*,*Trichophyton rubrum,Fusarium soleni,* and *Aspergillus niger*

Indian fish species viz. three Indian major carps, *Labeo rohita, Catla catla* and *Cirrhinus mrigala* was carried out for three bacterial strains ,*Trichophyton rubrum*,*Fusarium soleni,* and *Aspergillus niger* Antifungal activity of fish mucus was compared with blank sample.

Observations of skin mucus of fishes during mucus collection: Live specimen of length 19-34 cm and weight 190 - 910 grams of each species were selected for collection of mucus. Mucus was scrapped off at regular interval per month. Mucus secretion when compared on habitat basis (mid, surface and bottom dwellers) During the study when we compare mucus secretion according to their habits, it was also observed that secretion of mucus was higher in surface dweller fish *Catla catla* compared to *Labeo rohita* and *Cirrhinus mrigala and* selected fishes secreted more mucus during summer season as compared to rainy and winters. It was also observed that during breeding season mucus secretion was high and more viscous.

Mucus of *L. rohita*was clear and secreted moderate amount while mucus of *C. catla and C. mrigala secreted* viscous and concentrated mucus with bit yellowish tinge. In general, the mucus samples collected from the skin surface were in the form of sticky strands and had **characteristic visco-elastic properties. *Catla catla* secreted more mucus compare to other sample fish *C. catla> L. rohita> C. mrigala*.**

The mucus samples, when allowed to stand, get separated into two layers, a lower layer consisting of a compact mass, settled at the bottom and an upper fluid like moiety layer. *C.catla* secreted highest amount of viscous mucus as compared to other carps under study, while, *C.mrigala* secreted minimum amount and least viscous mucus. Mucus of *L. rohita* was watery

with yellowish tinge and a pungent odor. In general, the mucus samples collected from the skin surface were in the form of sticky strands and had characteristic visco-elastic properties. The mucus samples, when allowed to stand, get separated into two layers, a lower layer consisting of a compact mass, settled at the bottom and an upper fluid like moiety layer.

Comparison of the biochemical composition of skin mucus of fishes from different feeding habits and habitats: Carbohydrates and proteins are present in the mucosal layer of fish. The complement system is known to contain a category of protein and non-protein elements that contributes to both innate and adaptive immunity. It contains approximately 35 plasma and membrane-bound proteins; those conciliate a continuum of events for proteolytic reactions which results in the elimination of invading microorganisms (Boshra *et al.,* 2006).

Proteases: Proteases are a group of photolytic enzymes, which hydrolyse the peptide bonds present in proteins thereby converting them to shorter polypeptides and amino acids. Proteases in skin mucus of fish were reported for their key role in the natural resistance of fish against pathogens (Ingram, 1980) by directly acting on a pathogen or may indirectly prevent pathogen invasion by changing mucus texture to enhance the sloughing of mucus and detaching pathogens from the body surface (Aranishi*et al.,* 1998).

Esterase: Esterase is a hydrolytic enzyme that acts either individually or in conjugation with other immune substances in the mucus in resisting pathogens or in wound healing (Palaksha *et al.,* 2008). Fish mucus also contains a variety of PR which has a significant role in innate immune mechanisms (Subramanian *et al.,* 2007). PR cleaves bacterial protein and thus directly damaging the pathogen (Ingram, 1980). The physicochemical parameters were analysis in all the three seasons i.e. winter, summer and rainy season. Results of physical and chemical properties of water in different season are varied.

Lysozyme: Lysozyme is a representative endogenous antibacterial agent, which is also called as muramidase and catalytically hydrolyzes the bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the cell wall of bacteria to lyse bacteria. Lysozyme was detected from the skin mucus of numerous fish such as channel catfish *Ictalurus punctatus.* (Ourth *et al*., 1980) rainbow trout *Oncorhynchus mykiss* (Lie *et al*., 1989; Smith *et al*., 2000) and the ayu *Plecoglossus altivelis* (Itami *et al*., 1992).Proteases have been reported to exert antibacterial activity.

Qualitative and quantitative analysis of Protein in fish skin mucus: Protein concentration shows varied result in all the three species in winter, summer and rainy season. In winter season the protein concentration in *Catla catla* fish is found to be 578.553±49.94. The protein concentration was found to be maximum in winter season in *Labeo rohita* (970.98±99.47) and *Cirrhinus mrigala* (969.359±109.73) as compared to *Catla catla* in which it was found maximum in rainy season with the value 899.8287±62.56. In mucus of *Catla catla* minimum protein concentration in found in summer season with the value 322. 886±33.33. In mucus of *Labeo rohita* minimum protein concentration in found in rainy season with the value 498.434±26.91 and in *Cirrhinus mrigala* protein concentration is found to be minimum in summer season. Highest value of protein concentration is seen in the mucus sample of *Labeo rohita* in winter season and lowest value is found in *Catla catla* in summer season.

Concentration of Carbohydrate in Experimental fishes:

Carbohydrate: Wide range of carbohydrate concentration in µg/ml in the mucus sample of all the three species in three seasons was observed. The mucus of all the three experimental fish's shows maximum concentration of carbohydrate in winter season out of which the highest value belongs to *Labeo rohita* i.e. 970.98±99.47 and lowest value belong to *Catla catla* which is 39.80267±6.32. While mucus of *Labeo rohita* (28.747±3.5888) and *Cirrhinus mrigala* (32.80±6.38) shows minimum concentration of carbohydrate in summer season as compared to *Catla catla* (39.80±4.592) which shows minimum concentration of carbohydrate in rainy season.

Enzymatic test: The mucus sample of all the three fishes were tested for protease, esterase and lysozyme enzyme activities in three different seasons. Comparative result shows varied range of enzymatic activity in different seasons.

Protease: The protease activity of mucus sample of *Catla catla* is found to be maximum in summer season i.e. (1.4904±0.192) followed by winter season (0.0652±0.005) and rainy season (0.0493±0.0036). Similarly, maximum protease activity is found in the mucus sample of *Labea rohita* (1.533±0.152) and *Cirrhinus mrigala* (1.511±0.152) in summer season as compared to winter and rainy season. In *Labea rohita* minimum protease activity in seen in winter season i.e. 0.029±0.004 and in *Cirrhinus mrigala* minimum protease activity in seen in rainy season i.e. 0.0439±0.004.

Esterase: Esterase activity is found to be maximum (2.282±0.009) in winter season in the mucus sample of *Catla catla* followed by rainy season (2.281±0.0036) and summer season (0.236±0.0105). In the mucus sample of *Labeo rohita* esterase activity is found to be maximum (2.281±0.01) in rainy season followed by winter season (2.455±0.02) and summer season (2.281±0.01). Esterase activity is found to be maximum (2.642±0.057) in rainy season in the mucus sample of *Cirrhinus mrigala* followed by winter season (2.443±0.015) and summer season (0.462±0.011). In all the three experimental fishes summer season shows minimum esterase activity and the lowest value is seen in *Catla catla*.

Lysozyme Activity: Lysozyme activity in *Catla catla* and *Cirrhinus mrigala* species of fishes were found to be maximum in winter season, but minimum lysozyme activity is found in summer only in *Catla catla* and *Labeo rohita*. Minimum lysozyme activity in *Cirrhinus mrigala* is seen in rainy season.

Antifungal activity: Antimicrobial effect of the skin mucus of fish, Labeo rohita ,Catlacatla and *Cirrhinus mrigala* were tested against, pathogenic fungal strains viz, *Fusarium soleni* (MTCC No.-350), *Aspergillus niger*(MTCC No.-282), *Trichophyton rubrum* (MTCC No.-8477) in three different seasons (summer, rainy and winter). The activity was measured in terms of the zone of inhibition in cm/µL. The inhibition effect of mucus of all fishes against three pathogenic fungal strains in different seasons are given in tabulated. The zone of inhibition values of mucus was compared with the control.

Catla catla: Antifungal activity of mucus sample of *Catla catla* in winter and summer season was recorded maximum against *Aspergillus niger* which is 1.4 cm/µl and 11.5 cm/µl. But the antifungal activity of rainy season mucus sample was recorded against *Fusarium soleni.* Zone of inhibition is 0.9 cm/µl.

Labeo rohita: Seasonal variation of antifungal activity of *Labeo rohita* fish mucus against selected fungal species in winter season was recorded similar (0.6 cm/µl) for *Trichophyton rubrum* and *Aspergillus niger*. Zone of inhibition is recorded maximum in winter and summer season against both *Trichophyton rubrum* and *Aspergillus niger* fungi in the mucus sample of *Labeo rohita.* Maximum zone of inhibition is recorded against *Trichophyton rubrum* followed by *Fusarium soleni*, streptomycin and *Aspergillus* in rainy season.

Cirrhinus mrigala: Maximum zone of inhibition is seen in *Fusarium soleni*both in winter 0.7 cm/µl. and summer season 1.7 cm/µl. in the mucus sample of *Cirrhinus mrigala.* But in rainy season maximum zone of inhibition is found in *Trichophyton rubrum.* It is concluded the comparative tabulation that the mucus sample of *Catla catla* shows highest antifungal activity 2.7 cm/µl against *Trichophyton rubrum* in summer season which is also similar to the value of control. Lowest value is recorded in rainy season against *Trichophyton rubrum* and *Fusarium soleni* by mucus sample of *Labeo rohita* and *Cirrhinus mrigala.*

Table No. 03: Values of Different Parameter

Parameter	**Fishes**								
	C.catla			***L.rohita***			***C. mrigala***		
	Winter	**Summer**	**Rainy**	**Winter**	**Summer**	**Rainy**	**Winter**	**Summer**	**Rainy**
Protein(mg/ml)	578.55 ±49.94	322.88 ±33.33	899.82 ±65.56	970.98 ±99.47	612.61 ±76.68	498.43 ±26.91	969.3 ±109.73	583.7 ±33.37	787.35 ±44.334
Carbohydrate (mg/ml)	39.802 ±6.32	44.571 ±5.699	52±8	28.747 ±3.588	42.32 ±7.77	32.80 ±6.38	31.454 ±2.12	39.80 ±4.59	38.47 ±4.34
Enzyme essay									
Protease activity	0.0652 ±0.005	1.490 ±0.192	0.0493 ±0.0036	0.0291 ±0.004	1.533 ±0.152	0.0319 ±0.0039	0.0486 ±0.003	1.511 ±0.152	0.0439 ±0.004
Esterase activity	2.282 ±0.009	0.236 ±0.015	2.281 ±0.010	2.455 ± 0.021	0.498 ±0.019	2.281 ±0.010	2.443 ±0.015	0.462 ±0.011	2.642 ±0.057
Lysozyme Activity	0.616 ± 0.66	0.534 ±0.052	0.559 ±0.056	0.0408 ± 0.0033	0.0343 ±.0027	0.100 ±0.011	0.310 ±0.032	0.298 ±0.029	0.275 ±0.033

All values are Mean ± S.E of mean Means with different letters in the same row are significantly (P<0.05) different (Data were analyzed by Duncan's Multiple Range tests)

Table No. 04: Comparative Antifungal activity of fish Integumentary mucus in -Zone of inhibition (in cm/µL)

Fish	***Catlacatla***			***Labeo rohita***			***Cirrhinus mrigala***		
Fungal strain	**Winter**	**Summer**	**Rainy**	**Winter**	**Summer**	**Rainy**	**Winter**	**Summer**	**Rainy**
Control(Streptomycin)	0.7	2.7	0.5	0.5	1.8	0.4	0.5	1	1
Trichophyton rubrum	0.9	2.7	0.4	0.6	2.5	0.2	0.5	1.1	0.3
Fusarium soleni	0.7	1	0.9	0.5	1.9	0.2	0.7	1.7	0.2

Aspergillus niger	1.4	1.5	0.8	0.6	1.1	0.4	0.5	1	1

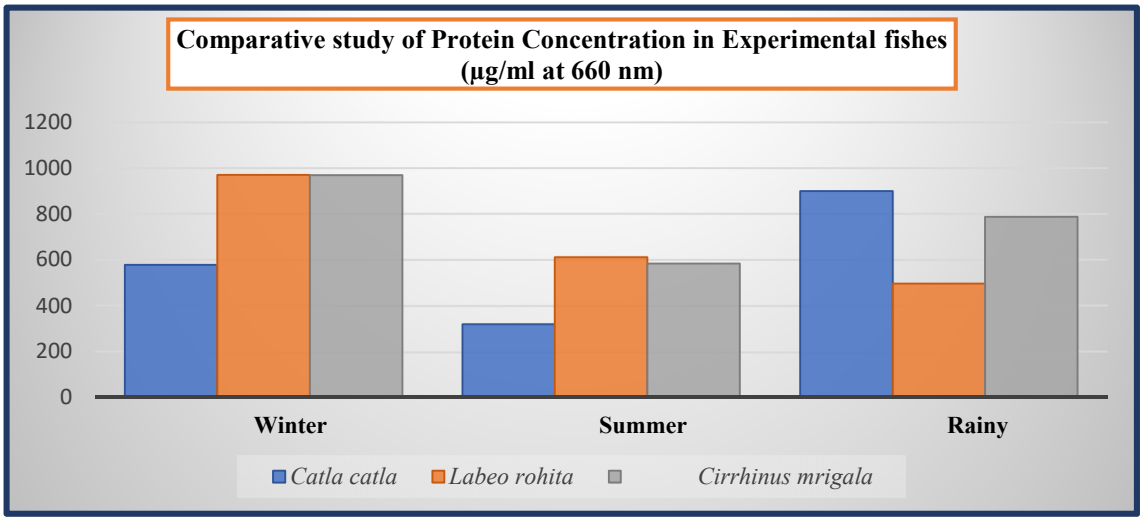

Fig. 02 Comparative study of Protein Concentration in Experimental fishes (µg/ml at 660 nm)

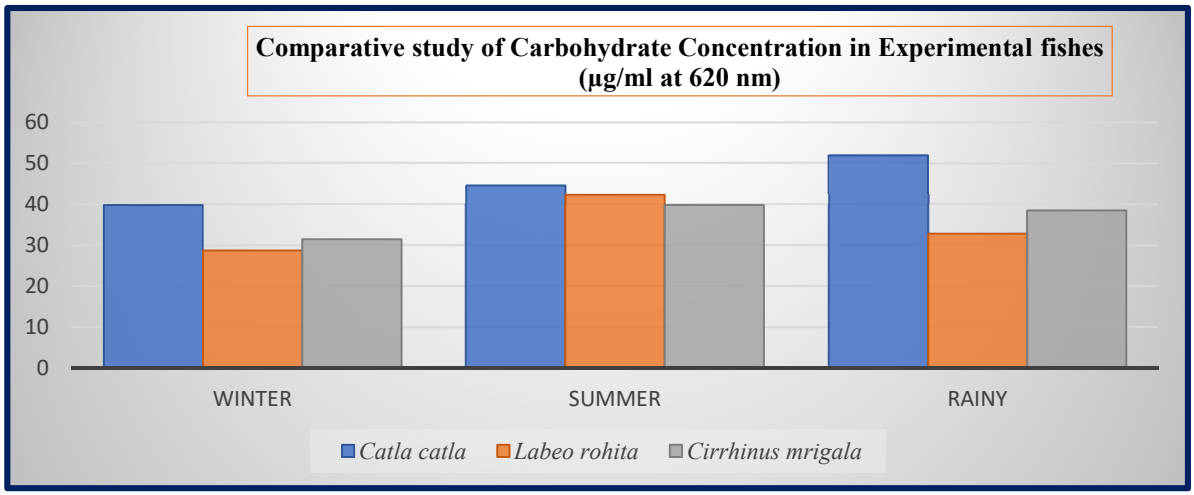

Fig. 03: Comparative study of Carbohydrate Concentration in Experimental fishes (µg/ml at 620 nm)

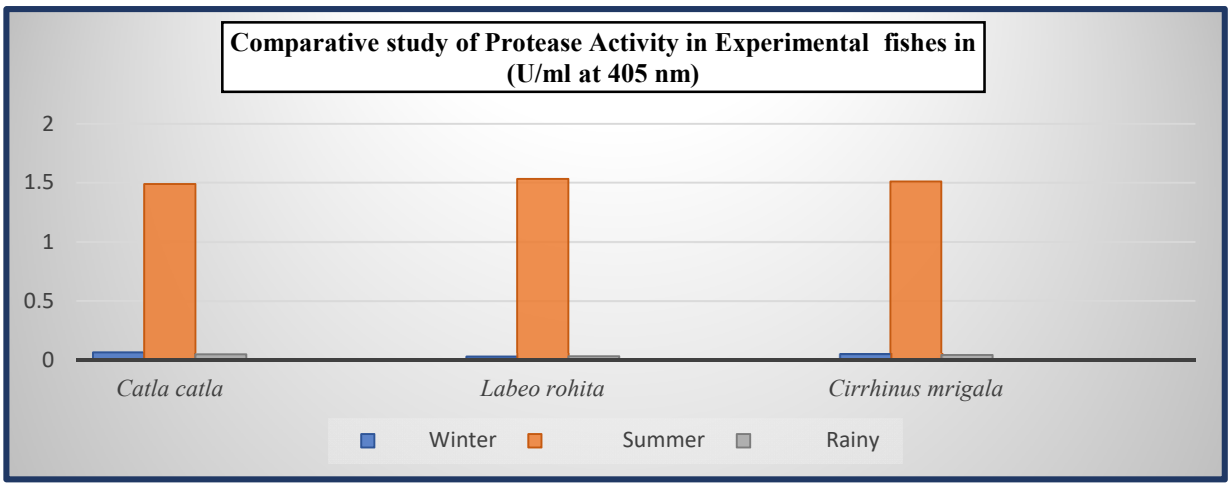

Fig. 04: Comparative study of Protease Activity in Experimental fishes in(U/ml at 405 nm)

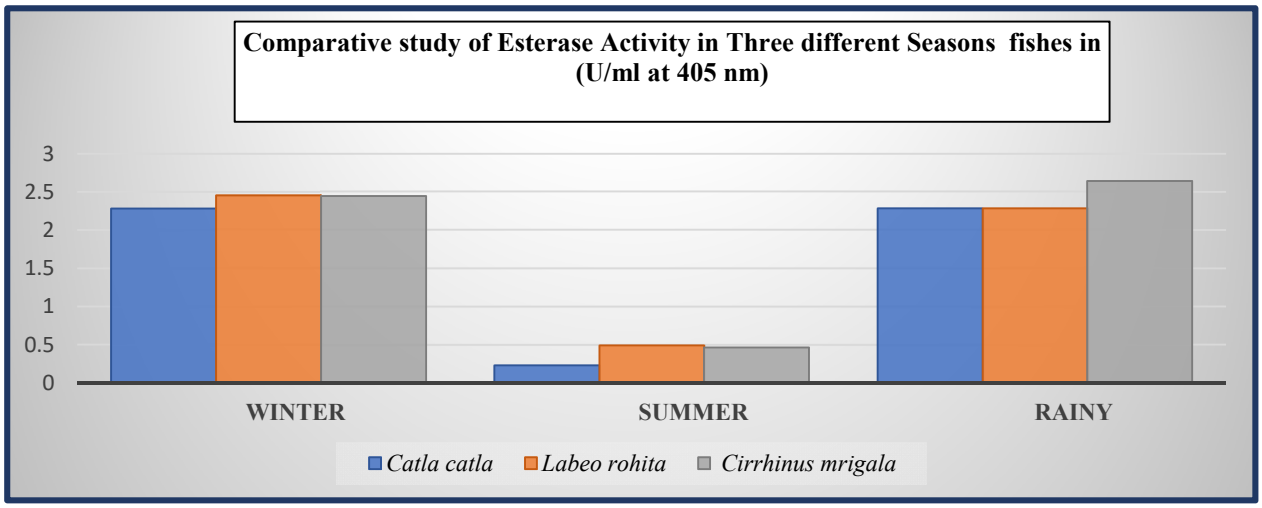

Fig. 05: Comparative study of Esterase Activity in Three different Seasons (U/ml at 405 nm)

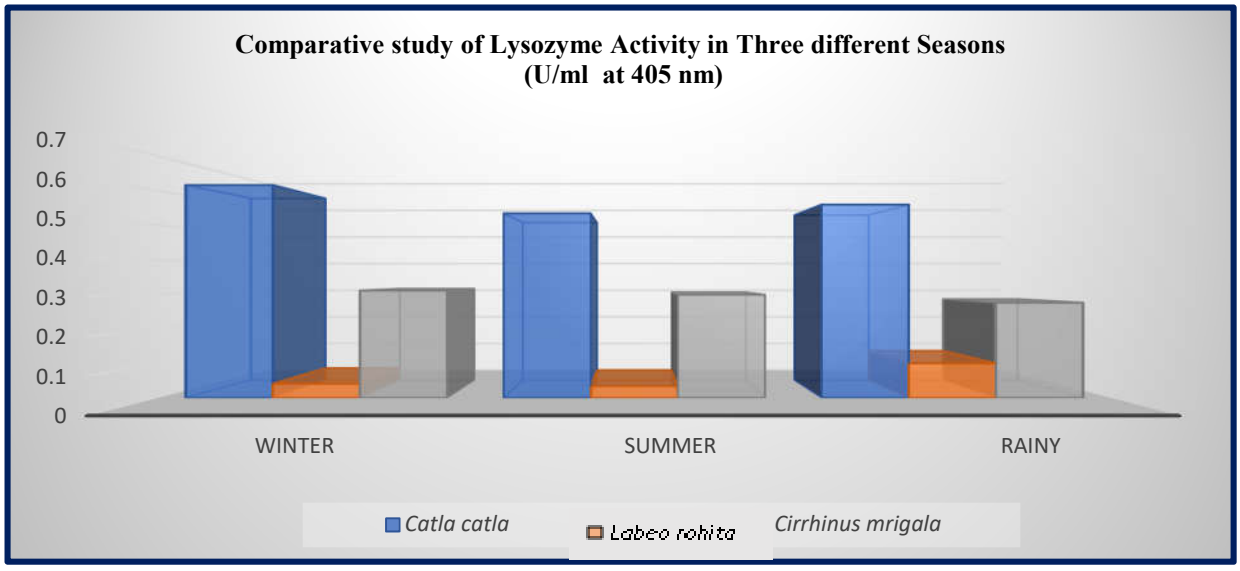

Fig. 06: Comparative study of Lysozyme Activity in Three different Seasons (U/mlat 405 nm)

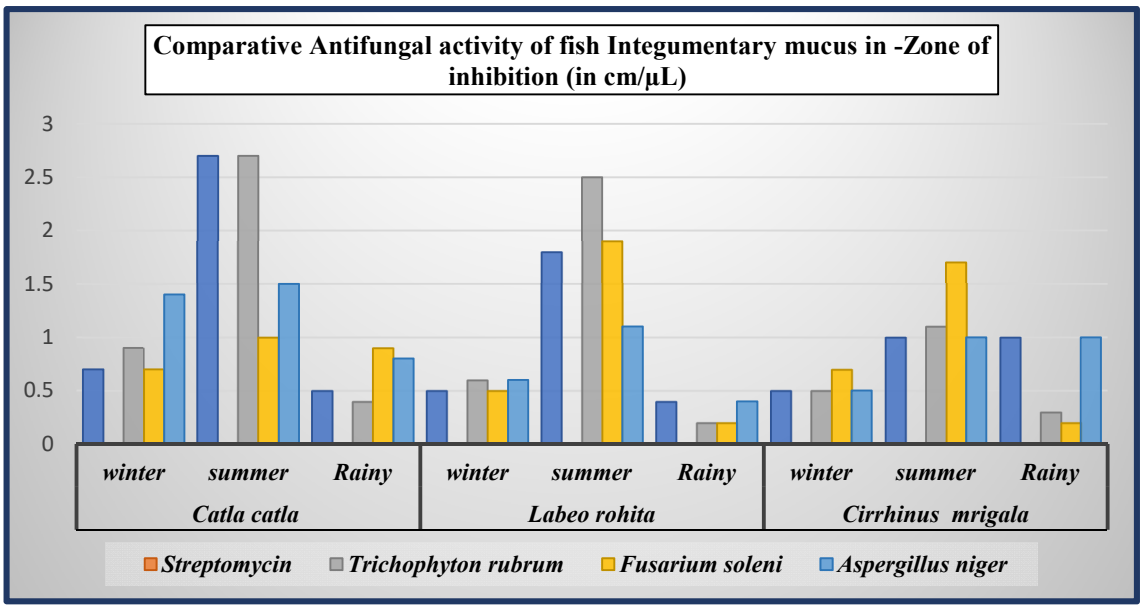

Fig.-6 Comparative Antifungal activity of fish Integumentary mucus in -Zone of inhibition (in cm/µL)

Conclusion: Recently, the integumentary mucus of the fishes has gained importance in the field of biomedical research, because of its ability to tackle infections caused by bacteria, viruses, and fungi, by providing innate immunity to the fishes. The present investigation suggested that the skin mucus of fishes is better sources of antimicrobial activity. It is being studied for its potential applications in human medicine. A detailed investigation is required for the specific immune responsible substance present in skin mucus of fish, by this way we design new medicines against various microbial diseases.

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