



## ANTIBACTERIAL EFFICACY OF INDIGENOUS AND EXOTIC CARPS MUCUS AGAINST COMMON PATHOGENIC BACTERIA

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### ABSTRACT

The trial was conducted to study the antibacterial efficacy of Indigenous (*Cirrhinus mrigala* & *Labeo rohita*) and Exotic Carps (*Hypophthalmichthys molitrix* & *Cyprinus carpio*) mucus against common pathogenic bacteria. All fish species of 300 to 350g were stocked and acclimatized for 15 days, weaned on commercial diet at 4% body weight. Before mucus collection fish were starved for 24 hours. Mucus secreted on epidermal surface of fish was collected in 5 attempts with time interval of 20 minutes. Bacterial strains i.e. *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli*, and *Staphylococcus aureus* were taken from the department of Zoology Government College University Faisalabad research lab repository. Ciprofloxacin was used as positive control. *L. rohita* secreted highest amount of mucus while least amount of mucus was secreted by *C. carpio*. Appearance of mucus of *L. rohita*, *H. molitrix* and *C. carpio* were highly viscous while *C. mrigala* was less viscous. Antibacterial activity of fish mucus shown that indigenous carps exhibited the best antibacterial activity as compared to exotic carps. Maximum antibacterial activity was observed by *L. rohita* mucus among indigenous carp. It showed more effect in controlling the growth of *P. aeruginosa* with zone of inhibition (23mm). These results showed that carp mucus play important role in protection of fish against the invasion of pathogens. Fish skin mucus can be utilized as an alternative of antibiotics which perhaps could be employed in aquaculture and also for humans. Being a natural product, therefore, it could help in reducing the problems of antibiotic resistance.

## 1. INTRODUCTION

Vertebrate organisms mostly included fish are the largest class of aquatic cold blooded organisms and its number is about half in vertebrate species that exist currently (Kumari et al., 2019). Fishes are the main protein source among aquatic life, providing 7% of entire protein supplies with 80% of the animal protein consumption. Per capita yearly fish consumption in Pakistan is around 1.9 kilogram which is lowermost contrasted to the advanced countries i.e. about 20kg (FAO, 2017). Pakistan's native carps like *Labeo rohita*, *Cirrhinus mrigala* and *Catla catla* are cultured in private and government sectors, also topmost the natural aquatic systems.

For the period of former twenty years cultivation of fish has been renowned as the endorsing world food industry (Yildirim et al., 2014). Fish are presented to a perplexing sea-going climate, calculating Minimum inhibitory concentration of Mucus which contains different sorts of pathogenic and nonpathogenic microorganisms. These are equipped for processing and corrupting of fish tissues. This has come about infections in fish. Compelling administration of these sicknesses is one of the most basic components for fruitful hydroponics. The support of huge quantities of fish in a little region gives a climate helpful for the turn of events and spread of irresistible ailments and because of the collection of homegrown waste in the amphibian medium, the number of inhabitants in organisms is expanded quickly (Wang et al., 2019).

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In swarmed, generally unnatural climate, fish are focused and more defenseless to infections. Besides, the water climate, and restricted water flow, encourages the spread of microorganisms inside clogged populaces. Issues of irresistible illnesses become more intense when fish are developed in high densities in concentrated hydroponics. Despite the fact that under ordinary conditions fish holds a solid state by utilizing their natural invulnerable frameworks that have discharged explicit mixes against the organisms tainted on them. This intrinsic immunological framework gives the main line of protection (Devi et al., 2019).

In fish the epithelial surfaces, of nutritious lot, skin and gill initially interact with irresistible operators and are secured with an oily layer known as mucus (Guardiola et al., 2017). The mucosa comprises of a cell and a humoral part. The cell part comprises of the mucous film and its hidden connective tissues and humoral part comprises of extracellular atoms present in the skin bodily fluid. Fish bodily fluid is discharged by epidermal cells and contains mucins and different substances, for example, inorganic salts, immunoglobulin, proteins and lipids suspended in water giving it trademark greasing up properties. Mucin assumes a significant function in wound mending. Mucin has expected antimicrobial and toxic properties (Rasheed et al., 2018).

This mucus layer thought to go about as grease. This layer wraps the outside of outer body to shield from injury and as a mechanical obstruction of the skin it ruins path in most of microorganisms into the body (Islam et al., 2014). Carp skin or bodily fluid is a decent wellspring of organically dynamic mixes for different restorative purposes. Composition of fish bodily fluid and its rheological properties are crucial for the upkeep of bodily fluid capacities. Bodily fluid surfaces are dynamic grids and their piece differs among fish species and with endogenous (sex and formative stage) and exogenous components (stress, water, temperature, pH and contaminations) (Reverter et al., 2018).

Expanded fish creation, can satisfy the requirements of the developing human populace in any case, unfortunately in the cultivating of fish a few parasites negatively affect the fish creation. These parasites may be bacterial, contagious, and viral and here and there helminthes and goes through about 45% demolitions in the fish cultivating yield. Bacterial illnesses go through a huge misfortune in the wild and refined fish and the bacterial sicknesses are the most well-known to happen among the irresistible maladies ((Kousar et al., 2019).

*Klebsiella pneumoniae* is discovered to be irresistible to different creatures including fish. *K. pneumoniae* is chiefly answerable for wide scope of diseases including drain, red inconsistency along the group of *Cyprinus carpio* (Dash et al., 2018). *Pseudomonas aeruginosa* is a Gram negative bacterium. Fish micro biota typically contains *P. aeruginosa*; anyway under exacting

conditions, for example, unhealthiness and congestion the microscopic organisms have gotten exceptionally crafty and pathogenic (Lamari et al., 2017). *Staphylococcus aureus*, gram positive are round formed microbes. It is likewise one of the normal foodborne microbes and is liable for more than 240,000 food borne ailments. Every year *S. aureus* disease alongside different species have been accounted for in marine fish (Zhu et al., 2018). *Escherichia coli*, is gram negative microbes present in food, climate and digestive system, everything being equal. There is a critical issue of antimicrobial resistance of these bacteria as long as it can act on living organisms (Poirel et al., 2018).

In the reaction of these undesirable consequences, certain chemicals are frequently used to cure these ailments of fish and the constant application of those chemicals would be resulted in multi resistance strains of microbes occurring in the water environment. In such situation, the knowledge of instinctive immune system of fish has been limited, because they are toxic and persist for a longer time in environment. Consequently, the introduction of new medicines is needed, that can kill pathogen growth and can kill them. Such drugs in the fish will minimize the economic loss in one way and will improve the aquatic environment, by limiting the application of chemicals in other way (Dawood et al., 2019).

Every species of fish takes its own specific propensity and residence, involve assorted kinds of water climate and ingests numerous sorts of the food, that may influence the amount of released bodily fluid and its synthetic constituents inside or among the species and may be useful in as a wellspring of various of safe response and components (Kumari et al., 2019). As of now, data with respect to the intrinsic insusceptibility boundaries of bodily fluid is accessible just for some business fish species. In any case, little is known with respect to the inborn insusceptibility boundaries of the epidermal bodily fluid of the monetarily significant Indian major carps, that is, *Labeo rohita*, *catla* and *Cirrhinus mrigala*. Hence, in this examination exertion was made to contemplate the antibacterial movement of two Indian major carps *Labeo rohita*, *Cirrhinus mrigala* and two Chinese carps *Hypophthalmichthys molitrix*, *Cyprinus carpio* against chose microscopic organisms (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*). These microorganisms were chosen on account of their importance in diseases and high mortality in fish (Islam et al., 2014).

The main objective of this study was to compare the mucosal immunity (antibacterial activity) of two indigenous carps (*Labeo rohita* and *Cirrhinus mrigala*) and two exotic carps (*Hypophthalmichthys molitrix* and *Cyprinus carpio*) against pathogenic bacterial strains,

and to find out the most pathogen resistance carp species by

## 2. MATERIALS AND METHODS

### *Experimental Fish*

#### *Stocking*

For the execution of experiment total 7 fish of each carp species (*C. mrigla*, *L. rohita*, *H. molitrix* & *C. carpio*) with 300-350g were bought, from Fish Hatchery Satyana road Faisalabad and stocked into cemented tanks.

#### *Acclimatization*

Before the commencement of this trial fish were acclimatized to the laboratory environments for about 15 days, fed on commercial diet (32% crude protein) at 4% of their live wet body weight once a day. The water quality parameters i.e. pH, dissolved oxygen (5-7ppm) and temperature were maintained day by day as per standard strategies (APHA, 1985). Water exchange and removal of fecal matter was done manually by siphoning at regular routine. 50% of the water was changed once daily (Islam et al., 2014).

#### *Mucus Collection*

After one week of acclimatization fish were starved for 24 hours and bathed with 4% of potassium permanganate (KMnO<sub>4</sub>) solution afore mucus collection. Out of seven fish three healthy fish of each carp species were randomly selected. Collection of skin mucus was done without giving any chemical or anesthesia to the fishes (Kumari et al., 2019). For half an hour before mucus collection fish was kept out of water in specimen tray and epidermal surface of each fish secreted mucus that was collected as sample (Patil et al., 2015). Mucus was scraped carefully from dorsal surface of the body by using a sterile spatula (in anterior-posterior direction) from head to tail. From ventral surface of the body mucus will not be collected to prevent intestinal and urogenital impurities.

From each fish of four species mucus was collected after regular intervals of twenty minutes in five tries. Mucus was collected individually from each species of four carps in sterile tubes (Balasubramanian et al., 2012). Then the collected samples were centrifuged at 5000 rpm for the time of 20 minutes and supernatant was put in storage at 4°C to prevent any bacterial growth (Kumari et al., 2019).

#### *Mucus Extract preparation*

By using an improved protocol of Subramanian et al. the acidic extract of fish mucus was prepared. A mixture of mucus and 3% acetic acid was prepared by using 1ml of each fish mucus with 0.5mL of acetic acid and put in a boiling water bath for 5 minutes. Then chilled in ice and homogenized for 30 seconds by using vortex shaker.

This acidic mixture was then centrifuged at 18,000 rpm for 35 minutes at 4°C. Then the micropipette was used to collect the supernatant in sterilized eppendorf tubes. A syringe with 0.45µm syringe filter was used to purify the mucus. The obtained filtrates were then emptied in sterilized eppendorf tubes and put in storage at 4°C in a refrigerator (Lee et al., 2020).

#### *Antibiotic Preparation*

For antibacterial assay ciprofloxacin was used as a positive control.

#### *Test microorganisms*

All bacterial strains inspected (4 bacterial strains, Gram-negative bacteria *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*, Gram-positive bacterial strain *Staphylococcus aureus*) were taken from the department of Zoology Government College University Faisalabad research lab repository.

#### *Agar Well Diffusion Method*

Agar well diffusion is extensively used to calculate the antimicrobial activity of fish skin mucus (Al-Rasheed et al., 2018).

#### *Antibacterial Assay*

##### *Procedure*

Nutrient broth media was prepared, by liquefying the nutrient broth with the amount of 2.5g using 100ml of the distilled water. Media was autoclaved for 15 minutes at 121°C and cooled for 40 minutes at room temperature. 5ml broth media was then poured into the autoclaved falcon tubes. The inoculum was then prepared by adding a pure colony of single bacteria using a streaking loop (sterilized) individually for each bacterial strain. Falcon tubes (containing bacterial strain and broth media) were then incubated over night at 37°C to allow the microorganisms to grow. On the very next day, 100µl of this inoculum was poured on the prepared nutrient agar plates using micropipette (with the addition of 2.8 g of nutrient agar in 100ml of distilled water, autoclaved for 15 minutes at 121°C and emptied in sterilized petri plates). After spreading inoculum, plates were kept aside for a small amount of time; in each plate six wells were made with the help of cork borer. The middle well was loaded with positive control ciprofloxacin and four sided wells were filled with mucus extract from 4 carp species and last one was filled with the negative control i.e. 3% acetic acid solution by using micropipette. Plates were labeled carefully and placed for few minutes to settle down the filled material. Afterwards, plates were incubated for 24 hours at 37°C. To ensure safety and sterilization protocols, all the procedure was done under laminar flow.

Each procedure was performed in triplicates. After the period of incubation, around each well zones of inhibition were measured by using a scale in mm. The

Zone of Inhibition portrays the mucus extract's bactericidal activity, also any resistance of examined bacterial strains against mucus extract. Average of three readings was calculated as its Zone of Inhibition (ZOI) (CLSI, Clinical & Laboratory Standard Institute, 2012).

#### **Evaluation of Minimum Inhibitory Concentrations (MIC) of indigenous and exotic carp mucus**

Minimal inhibitory concentration can be defined as the lowest concentration of antibacterial material that decreases the growth rate of bacteria.

MIC was measured using broth dilution method. Overnight cultures of *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, isolates were prepared in normal saline whose turbidity was in contrast with 0.5 McFarland standards. In 96 wells microtiter plate, 100µl of Muller Hinton broth was poured in all 12 wells. 100µl of crude mucus of *Labeo rohita* was poured into 1<sup>st</sup> well. The mucus was consecutively diluted up to 10<sup>th</sup> well of microtiter plate. Lastly 100µl of bacterial inoculum was distributed in each well till 10<sup>th</sup> well and then into 12<sup>th</sup> well. The 11<sup>th</sup> well act as positive control as it contained only broth. Well 12<sup>th</sup> act as negative control with broth and bacterial suspension. The plates were covered properly with parafilm and incubated for 18-24 h at 37 °C (Alekish et al., 2018). All of the reactions were performed in triplicates. After incubation for 18 to 24 h, absorbance of viability of bacterial cell was calculated at 650 nm by the ELISA Reader.

### **3. RESULTS AND DISCUSSION**

#### **Average quantity of mucus collected**

Highest average mucus secretion was observed in *L. rohita* (12.5ml) that was of weight 349.86g and length 25cm. Moderate average mucus secretion was observed in *H. molitrix* (9.2ml) that was of weight 330.90g and length 23.8cm. While Lower average mucus secretion was observed in *C. mrigala* (6.5ml) that was of weight 320.67g and length 23cm while lowest average mucus secretion was observed in *C. carpio* (4.8ml) that was of weight 307.80g and length 20cm (Table 1).

#### **Appearance of mucus**

Mucus of exotic carps *H. molitrix* was highly viscous in nature whereas, *C. carpio* was viscous and also sticky in nature similarly mucus of indigenous carps *L. rohita* was highly viscous and sticky in nature whereas, *C. mrigala* was frothy and sticky in nature but less viscous (Table 1).

#### **Comparison between antibacterial effect shown by Indigenous and exotic carps mucus against common pathogenic**

Our results showed maximum zone of inhibition with Carp mucus against all isolates as shown in figure 1.

#### **Against Gram positive bacteria *Staphylococcus aureus***

Indigenous carps (*Labeo rohita*) mucus revealed great antimicrobial activity against *Staphylococcus aureus* with growth inhibition zone 22mm in diameter and *Cirrhinus mrigala* showed inhibition zone 18mm in diameter. Exotic carp's mucus also revealed antibacterial activity against *Staphylococcus aureus*. *Cyprinus carpio* mucus presented inhibition zone of 16mm and *Hypophthalmichthys molitrix* mucus showed inhibition zone 18mm. Ciprofloxacin was used as positive control exhibited 24mm inhibition zone. Mucus of indigenous carps showed more antibacterial activity than exotic carps. *Labeo rohita* mucus showed higher antibacterial activity against *Staphylococcus aureus* (Table 2).

#### **Against Gram negative bacteria *Escherichia coli***

Indigenous carps *Labeo rohita* mucus showed highest antimicrobial activity against *Escherichia coli* with inhibition zone 22mm in diameter and *Cirrhinus mrigala* showed inhibition zone 15mm in diameter. Exotic carp's mucus also revealed antibacterial activity against *Escherichia coli*. *Hypophthalmichthys molitrix* mucus presented inhibition zone of 15mm and *Cyprinus carpio* mucus showed inhibition zone 13mm. Ciprofloxacin was used as positive control exhibited 20mm inhibition zone. *Labeo rohita* mucus showed higher antibacterial activity against *Escherichia coli* with zone of inhibition (22mm) than Positive control (20mm) (Table 2)

#### **Against Gram negative bacteria *Pseudomonas aeruginosa***

Indigenous carps *Labeo rohita* mucus showed highest antimicrobial activity against *Pseudomonas aeruginosa* with growth inhibition zone 23mm in diameter and *Cirrhinus mrigala* showed inhibition zone 19mm in diameter. Exotic carp's *Hypophthalmichthys molitrix* mucus also showed antibacterial activity against *Pseudomonas aeruginosa* with inhibition zone of 11mm while *Cyprinus carpio* mucus also presented inhibition zone of 11.5mm. Ciprofloxacin was used as positive control with inhibition zone of 22mm but *Labeo rohita* mucus found more effective (Table 2)

#### **Against Gram negative bacteria *Klebsiella pneumoniae***

Indigenous carps (*Labeo rohita*) mucus showed maximum antimicrobial activity against *Klebsiella pneumoniae* with growth inhibition zone 19.5mm in diameter and *Cirrhinus mrigala* showed inhibition zone 17mm in diameter. Exotic carp's (*Hypophthalmichthys molitrix*) mucus showed inhibition zone 10mm in diameter while *Cyprinus carpio* mucus presented

inhibition zone of 12mm against *Klebsiella pneumoniae*. Ciprofloxacin was used as positive control and more effective against *Klebsiella pneumoniae* with growth inhibition zone diameter 21mm (Table 2).

Means were compared by Tucky's Test using SPSS: Means sharing similar letter in uppercase are statistically non-significant ( $P>0.05$ ).

#### MIC assay

*Labeo rohita* mucus extract exhibited a strong antibacterial effect during ZOI's measurement so three different fishes of *Labeo rohita* was taken for its MIC determination using the broth micro-dilution method. *L. rohita* mucus extract showed different MICs against different selected microbial strains (figure 2).

The present study revealed that all the investigated indigenous (*C. mrigla* and *L. rohita*) and exotic carp species (*H. molitrix* and *C. carpio*) secreted huge quantity of mucus and the volume of secretion varied among the four species. Highest mucus secretion was observed in *L. rohita* (12.5ml) that was of weight (349.86g) and length (25cm). Moderate mucus secretion was observed in *H. molitrix* (9.2ml) that was of weight (330.90g) and length (23.8cm). Lower mucus secretion was observed in *C. mrigla* (6.5ml) that was of weight (320.67g) and length (23cm) while lowest mucus secretion was observed in *C. carpio* (4.8ml) that was of weight (307.80g) and length (20cm). It was observed that body weight and length has great impact on fish mucus secretion. These results are in accordance with the studies of Nigam et al. (2012) for different fish species viz. *Cirrhinus mrigala*, *Labeo rohita*, *Catla catla*, *Rita rita*, and *Channa punctate* secreted different quantity of mucus. These variations in mucus secretion may be attributed to the different ecological and physiological conditions.

In present work both the indigenous and exotic fish species have antibacterial activity against the bacterial pathogens. But the indigenous fish species *L. rohita* and *C. mrigala* showed higher antimicrobial activity than that of the exotic fish species *H. molitrix* and *C. carpio*. Our consequences are also resemble with Islam et al. (2014) who determined that Indian major carp *L. rohita* and *catla catla* mucus demonstrated much resistance to the microbes.

*L. rohita* mucus showed highest antibacterial activity against all bacterial strains. This can be justified as *L. rohita* has higher antimicrobial compounds in fish epidermal mucus than fish species studied. The difference of antimicrobial compounds in skin mucus in these fish species could be associated with the habitat and species specific variations. Moreover, this can be said that *L. rohita* is a hardy fish and can survive a varied range of environmental situations. These results are in accordance with Dash et al. (2014) revealed that epidermal mucus enzymes such as lysozyme and

proteases play a significant role in fish immunity against pathogens. In his work highest level of lysozyme activity was obtained in skin mucus of *L. rohita* ( $10.95 \pm 0.33$   $\mu\text{g/ml}$ ).

In this study dissimilarity in their antibacterial activity was observed among the fish mucus against different or same bacteria. Adel et al. (2018) argued that these differences might be due to the some genetic factors, sex, age, nutritional impacts stress level, variation in habitat, different ecological niches or even due to different laboratory conditions and procedures. Lim et al. (2018) also revealed that antimicrobial activities were higher in acidic solvents than in aqueous or crude mucus. *Labeo rohita* was taken forward for its MIC determination. Mucus extract of *L. rohita* showed MIC at 50 $\mu\text{l/ml}$  against *K. pneumoniae* and against *E. coli*, *S. aureus* and *P. aeruginosa* is 25 $\mu\text{l/ml}$ . The minimum concentration of mucus ranged between 25 $\mu\text{l/ml}$  to 50 $\mu\text{l/ml}$  was able to inhibit the growth of the selected bacterial pathogens. Rao et al. (2015) also studied the MICs of skin mucus extracts of *C. striatus*, *Dasyatissephen* and *Himanturagerrardi*, *Tilapia*, *C. nigrodigitatus* against several infectious microbes. Kumari et al. (2019) also studied the MIC in *H. nobilis*, Grass carp and gulfam. Showed MIC at 25 $\mu\text{l/ml}$  against *K. pneumoniae*, *E. coli* and *B. cerus* and 50 $\mu\text{l/ml}$  against *P. aeruginosa*, *S. epidermidis*, *S. aureus* and *A. hydrophila*. Antibacterial activities shown by carp species studied; clearly indicate the presence of antimicrobial compounds in fish epidermal mucus

Overall the study showed that fish mucus could be source of antimicrobial agent for human and fish pathogens.

#### 4. CONCLUSION

This work proves to be an excellent evidence of the medicinal value of the mucus collected from selected indigenous and exotic carps. Being a natural product, therefore, it could help in reducing the problems of antibiotic resistance and thus could prove to be a cost effective product. Further studies are needed to isolate the antimicrobial substances from the mucus of these cultivable fish species and the mechanism of antimicrobial action.

#### 5. CONFLICT OF INTEREST

All authors have declared that there is no conflict of interests regarding the publication of this article.

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**Table 1. Appearance and average amount of mucus collected from experimental carp species.**

Fish Species	Average Length(cm)	Average Weight(g)	Average Mucus(ml)	Viscosity and appearance of mucus
<i>L. rohita</i>	25	349.86	12.5	Highly viscous and sticky in nature
<i>C. mrigla</i>	23	320.67	6.5	Less viscous/frothy and sticky in nature
<i>C. carpio</i>	20	307.80	4.8	Highly viscous/sticky in nature
<i>H. molitrix</i>	23.8	330.90	9.2	Highly Viscous in nature

**Table 2. Comparison of means of Zone of inhibition by carp mucus against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.**

Fish Mucus	Concentration	Inhibition Zone (mm)			
		<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Klebsiella pneumoniae</i>
<i>C. mrigala</i>	2:1(mucus: 3% acetic acid)	20±2 <sup>AB</sup>	15±1 <sup>A</sup>	19±1 <sup>B</sup>	17±1 <sup>B</sup>
<i>C. carpio</i>	2:1(mucus: 3% acetic acid)	17.67±1.53 <sup>A</sup>	13.34±1.53 <sup>A</sup>	11.5±.5 <sup>A</sup>	11.67±1.53 <sup>A</sup>
<i>L. rohita</i>	2:1(mucus: 3% acetic acid)	22.34±1.53 <sup>BC</sup>	21.67±1.53 <sup>B</sup>	22.67±.57 <sup>C</sup>	22.67±.5 <sup>BC</sup>
<i>H. molitrix</i>	2:1(mucus: 3% acetic acid)	17.67±0.57 <sup>A</sup>	14.67±1.04 <sup>A</sup>	11.34±1.04 <sup>A</sup>	11.34±1.52 <sup>A</sup>
Control (Ciprofloxacin)	5mg/ml	24±0.00 <sup>C</sup>	20±0.00 <sup>B</sup>	22±0.00 <sup>C</sup>	22±0.00 <sup>C</sup>

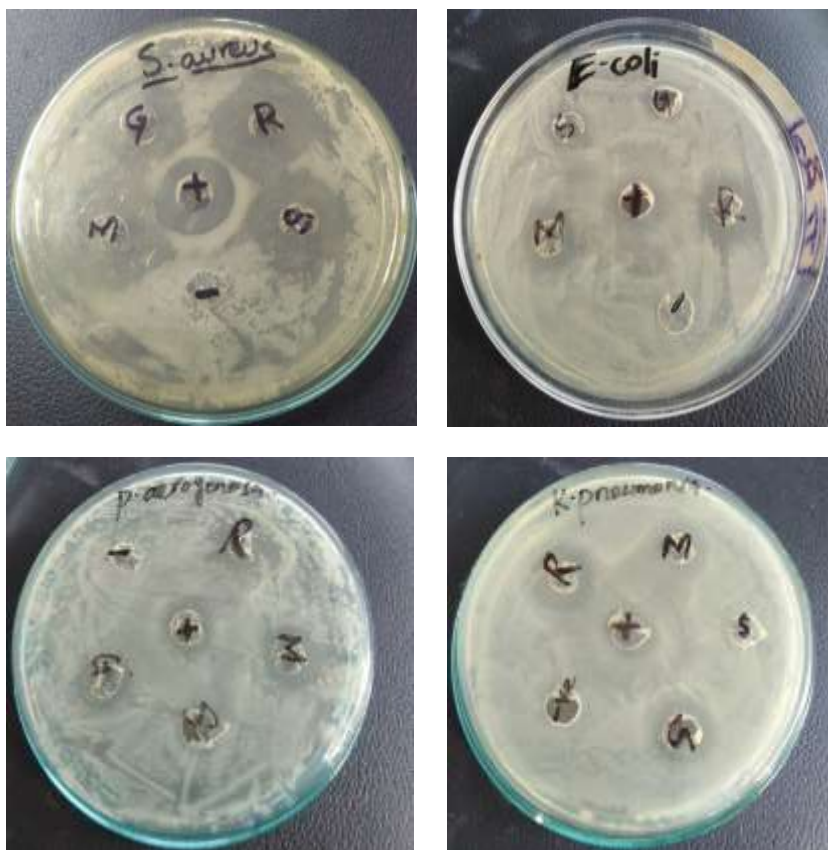


Figure 1. Zone of inhibition of Indigenous and Exotic carp's mucus against the growth of *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae*.

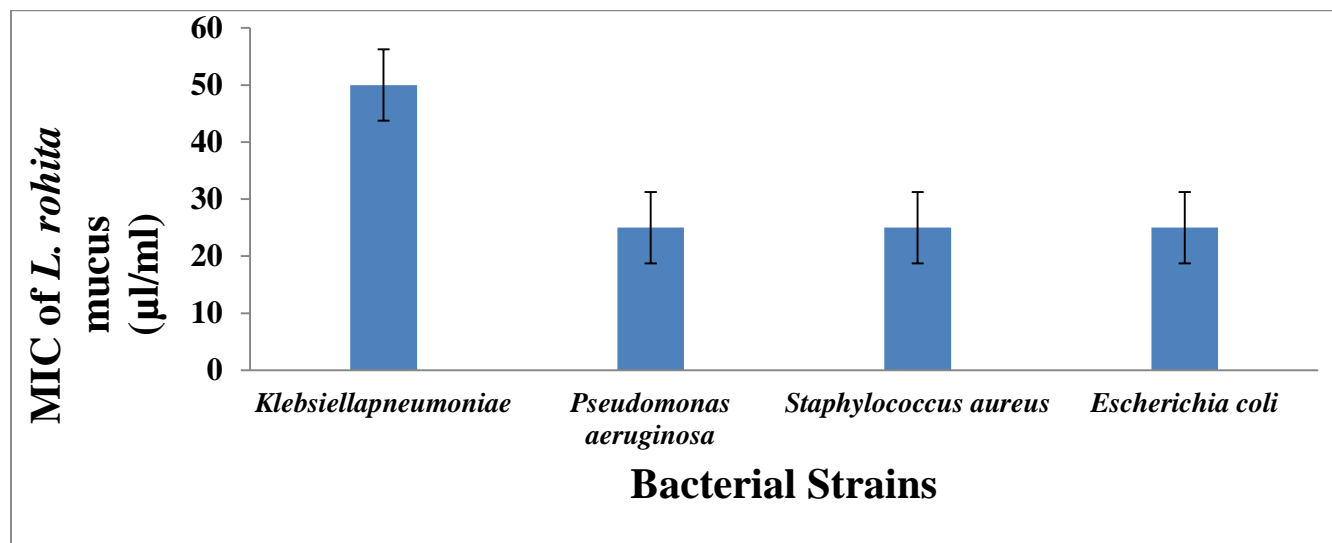


Figure 2. MIC shown by *Labeo rohita* against all bacterial strains.