Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env.Pharmacol. Life Sci., Vol 11[10] September 2022 : 66-71

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Environmental pollution of laundry detergent affects the first-line defence mechanism in *Chirrinus mrigal* and *Oreochromis niloticus*

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ABSTRACT
Antimicrobial agents found in fish epidermal mucus serve as the first line of defence against invading pathogens. The current study aims to evaluate the antimicrobial properties of Chirrinus mrigal and Oreochromis niloticus epidermal mucus against Vibrio harveyi and Staphylococcus aureus when exposed to different laundry detergent concentrations. The mucus content of the fish was extracted, then exposed to the LC50 of laundry detergent to determine its quantity and antibacterial activity.The results showed that the mucus secretion by these two fishes' epidermal skin increased with increasing detergent concentration in the water; however, the activity of lysozyme and alkaline phosphatase, as well as the bactericidal property of the epidermal mucus, were severely affected against the selected bacterial strains. Thus, laundry detergent contamination has a significant impact on the innate immunity of fish species, thereby affecting the health of aquatic organisms.
Keywords: *antibacterial activity, aquatic toxicology, fish immunity, lethal concentration, sodium lauryl sulphate,*

Received 14 .08.2022 Revised 22.08.2022 Accepted 13.09.2022

INTRODUCTION

Because of industrialization and urbanisation, the production and use of synthetic chemicals for cleaning purposes in homes, industrial premises, pesticide formulations, and dispersing oil spills at sea has increased globally.[1–2] There are two types of detergents commonly used for cleaning: phosphate detergents and surfactant detergents. Surfactants, for example, are extremely toxic and contain linear alkylbenzene sulfonate (LAS).[3] The majority of soap ingredients are biodegradable, but detergent ingredients are not and are produced from cheap petrochemical sources.[4] After usage, these detergents are drained into the aquatic environment thereby resulting in eutrophication and burnt effect on the diversity of plankton. Most of the detergent remains in the aquatic system for a long periodof time due to their slow degrade nature affecting various vital organs of the aquatic organisms through the food chain. [3] It alsohas been reported that surfactant increases microbial population in the aquatic medium.[5,6]
Fishes are the most important non-target aquatic organism affected by detergent pollution. They live in microbe-infested environments and have developed an effective defensive mechanism through their epidermal mucus, secreted in the skin.Teleost skin secretes mucus which is involved in immune functions.[7] The mucus is extremely important as the first line of defence against the invasion of environmental pathogens.[8]
The fish skin mucus contains several antimicrobial factors [9] and thus becomes an important component of the innate immune mechanism in order to provide a first physical and chemical barrier against pathogens and also, its various other vital activities like maintaining osmoregulation, chemical communication, swimming performance. Only a handful number of studies have been done so far dealing with the impact of detergents on biochemical, haematological, histological and biomarker aspects of fishes.[7, 10, 11] However, very few reports are available on the secretion of epidermal mucus and its antimicrobial property in fish, exposed to commercial detergents.
Due to the raising concerns with detergent pollution in urban water bodies and the diseases in fish culture, the present study is aimed to evaluate the impact of detergents on mucus secretions and their

bactericidal property in two freshwater fishes, *Oreochromis niloticus* (Linnaeus 1758) and *Cirrhinus mrigala* (Hamilton, 1822).

MATERIAL AND METHODS

Collection and Maintenance of Fishes

Live specimens of two freshwater fishes (*O. niloticus*and*C. mrigala)*, with an average weight of 10±0.3g and length of 15±0.2cm, used in this study, were collected from the Fisheries Research and Information Centre (FRIC) (Inland), Hebbal, Bengaluru, due to their feeding availability, and the mucous secreting ability. The collected fishes were acclimatized for 2 weeks in the aquarium separatelyin the aqua lab of CHRIST (Deemed to be University), Bengaluru and were transferred to 20 L aquarium tanks.To maintain hygiene and water quality, about half of the water was changed daily. The health of the fish was observed on a daily basis and the dead fish or fish with lesions, if any, were removed from the tank.During acclimatization, the fishes were fed with commercial pellet feed with 28% protein (Godrej feeds),twice a day at 4% of their body weight.

Preparation of detergent

The laundry detergent power was purchased from a local hypermarket, in Bengaluru. The detergent was weighed and dissolved in water to get the desired concentrations of 10, 20, 30, 40 and 50ppm. The solution, then, was filtered and kept in sterile dark bottles (500 ml) in a cool environment (4 °C) until use.

Experimental Design

A total of 180 fishes (both tilapia and mrigal) were used for the present experiment. After acclimatization, the fish were randomly selected and divided into control (C) and five experimental groups of 30 fish (10 fish of each group, with 3 replicates) to determine the lethal concentration of 50% (LC_{50}). The control and experimental groups were denoted as C and E_1 to E_5 respectively. The control (C) group fishes were reared with normaltap water, without any addition of detergent; whereas the experimental groups (E_1, E_2, E_3, E_4 and E_5)were reared in the same water with different concentrations of detergents such as 10, 20, 30, 40, 50ppm respectively. The fishes were starved for 24 h before the detergent exposure treatment and also during the experimental period of 96h. The percentage of mortality of the fish was observed at every 24 h interval. LC_{50} value was calculated by using PROBIT analysis.

Collection of epidermal mucus

After determining the median lethal concentration of the detergent for each experimental group,(*O. niloticus* and *C. mrigala*), the fishes were exposed for 48 h to different ratios of their respective LC_{50} values, such as 0, 10, 50 and 100%for mucus collection. Before the collection of mucus, the fishes were washed with a 4% potassium permanganate solution. No aesthesis was given to the fishes for the collection of skin mucus. The mucus was collected from 8 fishes by scraping the tail, fins and dorsolateral surfaces of the body by moving sterile spatula in the anterior to posterior direction and the mucus sample collection was carried out at a regular interval of time (10 times a day).[12,13] The mucus collection from the ventral side was avoided to eliminate intestinal and urinogenital contamination. The collected mucus samples were labelled accordingly and stored at 20^0C.

Preparation of aqueous mucus extract

The epidermal mucus samples of both control and experimental fishes of *C. mrigal* and *O. niloticus* were collected and homogenised separately using a well-sterilized mortar and pestle by adding sterile 0.85% NaCl. The homogenate was centrifuged at 10,000rpm for 10 minutes at 4°C. The obtained clear supernatant was collected and filtered using Whatman No:1filter paper. This filtrate was stored at 4°C until used for the antibacterial assay.[14,15]

Enzyme activities assay

The activity of Lysozyme and Alkaline phosphatase were determined by following the methods of Ross et al., [16] and Subramanian et al., [17] respectively to study the effect of detergents on the innate immunity of the given experimental animals.

Antibacterial assay

The Agar well diffusion method was adopted to evaluate the antibacterial activity of the epidermal mucus extract collected from the control and experimental group of fishes against the selected bacterial strains.[15]Sterile Petri plates containing 20 ml of molten Muller Hinton agar was seeded with 24 h cultures of approximately 107 CFU ml-1 bacterial strains. The bacterial cultures were seeded on the surface of solidified agar by the swabbing method. The wells were then made aseptically (with a diameter of 6–7 mm) using a sterile 1ml micropipette tip. To this 100µl of fish epidermal mucus extracts from each experimental group were added to the respective wells.

To the aqueous extract, 0.85% NaCl (C1), and detergent water (without mucus) (C2) were also loaded as the control.The plates were then incubated for 24 h at 37°C.The diameter of the zone of inhibition (ZOI)

around each well was measured in millimetres (mm) including the well, to determine the bactericidal effect.[18]

Data analysis

Finney's PROBIT analysis was used to calculate the median lethal concentration (LC_{50}) of the detergent on fishes by the percentage (%) of mortality was observed for 96 h of exposure, using IBM SPSS Statistics 25.0 software at a 95% confidence limit. The antibacterial activity of skin mucus from both fishes was represented graphically using MS Excel.

RESULTS

Toxicity effect

During the period of toxicity tests, no mortality was observed in the control group. Whereas, in the experimental groups, the mortality rate was observed to be the higher rate in*C. mrigala* compared to *O. niloticus*. The results revealed that *C. mrigala* is more sensitive to the detergent than *O. niloticus*.The mortality rate was on the rise with the increasein detergent concentration (Table 1). Apart from the mortality, rapid movement, swimming, and instability were also observed in the experimental group of fishes. The sacrificed fish's organs, such as the pupils, fins, and gills were also examined. The organs were found to be pale incolour and a mucus layer was also observed on these organs (personal observation).

The median lethal concentration (LC_{50}) values of the detergent for 96 hr of exposure for both species of fishes were also recorded. The PROBIT analysis (in SPSS where the logarithmic base is 10 with 95% significance) revealed that the LC_{50} of the detergent on *C. mrigala* and *O. niloticus*for an exposure period of 96 h was found to be 30.92ppm and 42.0ppm respectively (Table 1).

Impact of detergent on the secretion of epidermal mucus

The experimental group of fishes exposed to detergents were found to contain high mucus secretion, compared to the control group of fishes and was found to be increasing with the increased concentration of detergents. The control *C. mrigala*and *O. niloticus* secreted 0.5 and 0.4ml of mucus respectively, wherein the secretion of mucus was on to a higher note in the experimental group of fishes, accountingfor0.75mland 1.2 ml in *C. mrigala* and *O. niloticus* respectively. The mucus secretion was again found to be higher in *C. mrigala*than *O. niloticus*. The nature of mucus produced by tilapia fish was off-white in colour, watery in appearance and less viscous in nature.

Enzyme activity assay

Both lysozyme and alkaline phosphatase were present in the mucus samples of control fishes. However, pronounced variations in the level of enzyme activity were observed in the detergent exposed fish mucus. The results revealed that the activity of lysozyme and alkaline phosphatase were reduced significantly.

Antibacterial assay

The epidermal mucus samples, collected from the control and experimental group of fishes of *C. mrigala* and *O. niloticus*, were subjected to bactericidal activity, along with 0.85% NaCl and detergent water of LC_{50} value solution as a control set, against *S. aureus* and *V. harveyi*. The results revealed that the mucus collected from the control group of fishes registered strong antibacterial activity. The mucus of *C. mrigala* and *O. niloticus* showed the zone of inhibition (ZOI) of 21.73±0.06 &18.53±0.05mm and 22.80±0.35 &19.93±0.31mm against *S. aureus* and *V. harveyi* respectively.

On the other hand, the bactericidal activity of epidermal mucus produced by both the experimental group of fishes reduced significantly and registered low antibacterial activity with the increasing concentrations of the detergents used. The ZOI of both *C. mrigala* and *O. niloticus* mucus were maximum when they were exposed to 10% and minimum at 100% of their respective LC_{50} values. However, the bacterial property again was found to be higher in the skin mucus of *C. mrigala* than *O. niloticus* (Table 3). It is interesting to mention that the mucus of *C. mrigala* can effectively eradicate *S. aureus* than *V. harveyi*, whereas a reverse result was observed with the skin mucus of *O. niloticus* (Fig: 1&2).

Table 1: Cumulative Mean % of mortality at different concentrations of detergent during the 96 hof exposure

Detergent cconcentration (ppm)	% Mortality of *C. mrigala*				% Mortality of *O. niloticus*			
	Duration of exposure (in hr)							
	24	48	72	96	24	48	72	96
0	0	0	0	0	0	0	0	0
10	0	0	10	10	0	0	0	0
20	0	10	20	30	0	0	0	10
30	20	30	40	40	0	0	20	20
40	30	30	40	60	20	30	30	40
50	40	60	80	80	40	50	60	70

Table 2. Zone of inhibition shown by aqueous epidermal mucus extract

Bacterial strains	Zone of inhibition by *C. mrigala* mucus (mm)				Zone of inhibition by *O. niloticus* mucus (mm)			
	Concentrations of aqueous epidermal mucus extract							
	0%	10%	50%	100%	0%	10%	50%	100%
V. harveyi	18.53 ± 0.05	12.03 ± 0.25	9.93 ± 0.31	7.67± 0.42	22.80 ± 0.35	21.90 ± 0.75	15.60 ± 0.40	12.77±0.40
S. aureus	21.73± 0.46	18.33 ± 0.35	15.87 ± 0.51	15.23 ± 0.32	19.93±0.31	14.63 ± 0.40	14.40 ± 0.53	9.97 ± 0.35

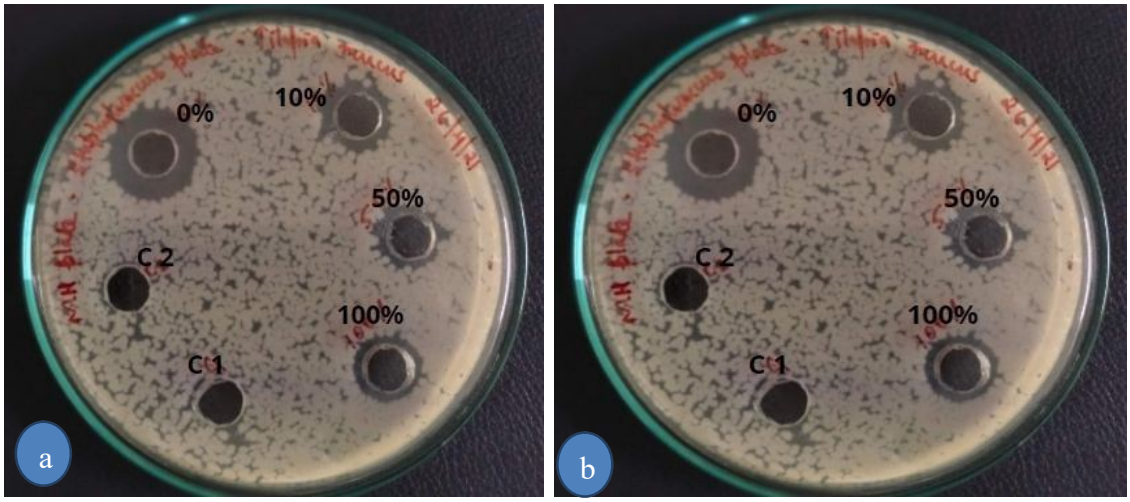

Figure Figure 1: Zone of inhibition shown by aqueous epidermal mucus extract of *O. niloticus* of all experimental groups against tested pathogenic bacterial strains, **(a)***Staphylococcus aureus*. **(b)***Vibrio harveyi*

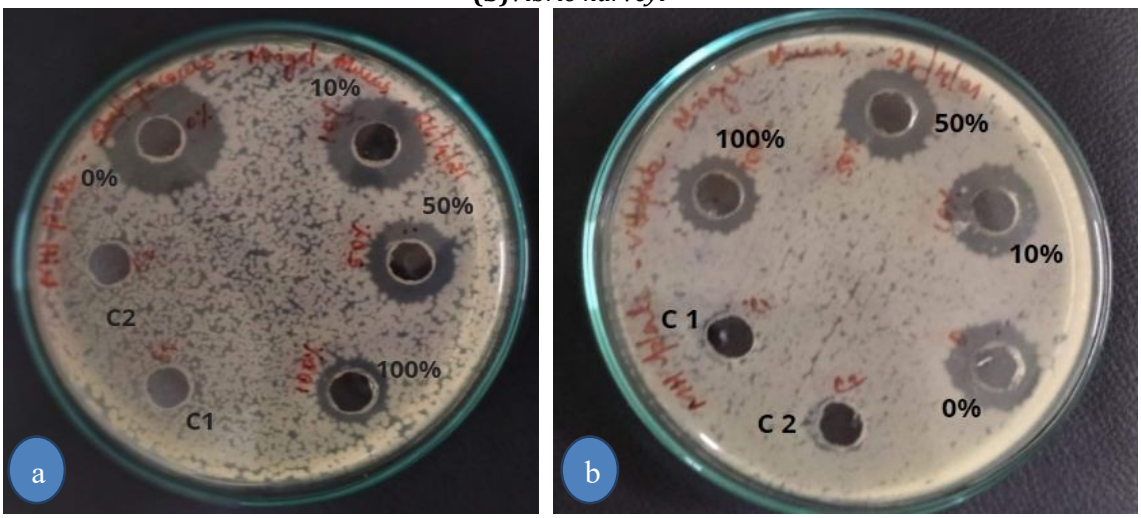

Figure 2: Zone of inhibition shown by aqueous epidermal mucus extract of *C. mrigala* of all experimental groups against tested pathogenic bacterial strains, **(a)***Vibrio harveyi***(b)** *Staphylococcus aureus*

DISCUSSION

In fishes, the increased mucus secretion may be considered as an indicator of increased stress level and activation of their innate immune system against various environmental flusters, pollution, ecological niches, bacterial stress etc.In the present study, the investigated freshwater fish species, *C. mrigala* and *O. niloticus* showed a high amount of mucus secretion when they were exposed to the detergents and the volume differed among the two different species. It can be inferred that the variations in the mucus secretion in different species may be due to the variations in their physiological conditions as well as due to the presence of detergent in the aquatic environment that affects the mucus-producing cells in their skin layers *ie.,* goblet cells.[13,19,20]Secretions of high mucus content were also reported in the earlier studies due to bacterial infections.[13,20-22]

The fish skin mucus is predominantly protein in nature.[11,23] The skin mucus layer of the fish surface performs many formidable functions, including disease resistance, respiration, ionic and osmotic

regulation, locomotion, reproduction, communication, feeding and nest building.[19]The protein content, along with other biochemical substances vary in different species due to variation in their physiology of growth, immunity, bacterial infections and also due to various environmental factors like the presence of pollutants etc. In the present study, the bactericidal property of the epidermal mucus of control fishes was found to be higher which confirms that fish mucus is a source of antimicrobial products. This functional aspect is defined by the gel-forming ability which is dependent on the mucus size, volume and cross-linking that occurs between mucin. The invading pathogens are entrapped in this mucosal layer present on the surface of the skin before making an entry inside. However, the skin mucus of the fishes exposed to the detergent showed very less antibacterial activity and this may be due to the damage or destruction of the mucus-secreting cells caused by the presence of detergents in the aquatic medium.[24,25]The presence of alkaline proteases as well as other detergent-compatible enzymes such as the lipases and amylasesused in the detergent industries to break down various stains during fabric washing,damage the skin surface of fishes.[26-29] The fish skin mucus is also more used in dermatological studies to overcome skin-related anti-infection defence mechanisms and to study the possible futuristic clinical applications. Since fish skin acts as a physical, chemical, and mechanical barrier to inter-individual communication, it maintains osmotic equilibrium and sensory functions by using visual signals such as pigments.[15]
Exposure to the detergent will cause stress to fish and other aquatic animals. The epidermal mucus produced by fishes in high detergent concentration was more in volume, on the other hand, the antibacterial activity shown by them was found to be less than mucus produced by control fishes (fishes at '0' detergent concentration). The presence of CF-14 an antimicrobial peptide (AMP) in the skin mucus of fish is the reason for the inhibition of bacterial growth.[14] Skin mucus got lesions due to the deterrent effect that might cause denaturation of antimicrobial peptides and enzymes present in skin mucus thereby reducing its antibacterial activity. Due to this, the fishes are more prone to pathogens.[20]

CONCLUSION

Thus, it is concluded that the presence of laundry detergents in the aquatic medium has a significant toxic effect on freshwater fishes *O. niloticus* and *C. mrigal*, by affecting the mucus secretory activity and also affecting their native defence mechanisms against the pathogens. Henceforth, the water should be properly treated before being discharged into the aquatic environment.

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CITATION OF THIS ARTICLE

M Raju, K Velayudhannair, M Pappuswamy, Nidhin M, John Paul A. Environmental pollution of laundry detergent affects the first-line defence mechanism in *Chirrinus mrigal* and *Oreochromis niloticus* . Bull. Env. Pharmacol. Life Sci., Vol 11 [10] September 2022: 66-71.