POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 36(3): 321–332, Y. 2021

ANTIBACTERIAL ACTIVITIES AND SUSCEPTIBILITY PATTERN OF BACTERIA ISOLATED FROM FISH MUCUS TO SELECTED CLINICAL BACTERIA ISOLATES

***Abidemi Esther Ojo*[1], *Thaddeus O. Ariom*[2], *Olugbenga Anthony Ojo*[3], *Seun Owolabi Adebajo*[4], *Abiola Tosin Oladotun*[5], *Sherifat Arike Opaleye*[6], *Nimotallahi Rabiu*[7]**

[1] ORCID: 0000-0001-6359-4086
[2] ORCID: 0000-0002-2959-8507
[4] ORCID: 0000-0001-7981-2965
[5] ORCID: 0000-0002-4592-7585
[6] ORCID: 0000-0002-1885-9759
[7] ORCID: 0000-0001-5508-1280
[1,2,4–7] Department of Microbiology
Federal University of Agriculture, Abeokuta, Nigeria
[3] Department of Pharmacognosy
Madonna University, Elele, Port-harcourt, River-state, Nigeria

Key words: fish mucus, antibacterial potentials, susceptibility pattern, spot-on-lawn assay and resistance.

Abstract

Fish mucus is known for its antimicrobial activities offering protection to fishes from environmental pathogenic attack. This study evaluates the antibacterial potentials of bacteria isolated from fish mucus of *Chrysichthys nigrodigitatus* and *Solea solea* on four clinical isolates: *Escherichia coli*, *Bacillus subtilis, Staphylococcus aureus* and *Aeromonas hydrophila* using the spot-on-lawn method and modified disc diffusion method. Susceptibility pattern of fish mucus bacterial isolates was determined using disc diffusion method. *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, *Citrobacter freundii* and *Shigella sonnei* were the isolates from the fish mucus varieties. Using spot-on-lawn assay, *Proteus mirabilis* exhibited the highest antibacterial activities against *Bacillus subtilis* (4.00±1.73 mm) while on the modified disc diffusion assay, *Shigella sonnei* exhibited highest antibacterial activities of (7.00±0.21 mm) against *Aeromonas hydrophila*. Among the isolates, only *Klebsiella pneumoniae* shown resistance to most of the conventional antibiotics used. Hence, bacteria isolated from fish mucus possess some antibacterial properties against clinical bacterial isolates.

Address: Ojo Abidemi Esther – Department of Microbiology, Federal University of Agriculture, Abeokuta, Nigeria. Email address: ojoae@funaab.edu.ng

Introduction

Fish is considered an efficient source of important nutritive protein that nourishes the body and hence consumed globally for this health benefits. Of all diet with a large reasonable amount of protein, fish stands out with closely 16% (world's population) animal protein (FAO 2007) and 17% in Africa population (ALLISON 2009). Microbiologically, fish and its bye products are of potential health risk due to its ability to harbour important human pathogenic bacteria. Bacterial infections can occur due to unhygienic handling and eating of poorly prepared ready-to-eat fish. Numerous bacterial genera such as *Escherichia*, *Listeria*, *Pseudomonas*, *Klebsiella*, and *Salmonella* have been identified from fish indicating several means of contamination of fish environs (MANHONDO et al. 2018, SICHEWO et al. 2014).

Fishes are naturally endowed with high immunological tolerance. One of the major line of defense exhibited in fishes is the skin slime that serves as first body soldiers (VENNILA et al. 2011). The mucus in the skin of fish naturally prevents invasion of most pathogenic microbes (includingand fungus) into the body of the fishes (VENNILA et al. 2011). Mucus of the fish is extensively released by the goblet cells (present in the skin) and it exclusively consists of mucins and other substances like inorganic salts, immunoglobulin, proteins and lipids (TYOR and KUMARI 2016). The mucus of *Claris* spp. have been explore in ages for its use in orthodox medicine to rejuvenate wounds, burns and tumor (CHINWUBA et al. 2016, DESLOUCHES and DI 2017). While *Anguiil abengalensis* has been in continuous use for the therapy of anaemia, burn injury, piles, and weakness in the Indian traditional medicine (RAHMAN et al. 2014), C*hanna striatus* is popular due to its ability to speed the rejuvenating rate of wounds, build up immune system and anti-inflammation including tender antifungal and antibacterial roles (WEI et al. 2010). These good roles have closely been associated to the presence of antimicrobial peptides (AMPs), polyunsaturated fatty acids (PUFA), mycosporine-like amino acids (MAAs) and organic acids (NWABUEZE and CAMPUS 2014).

Global daily increase in antimicrobial resistance to most commonly used antibiotics necessitated further attempts to search for novel antimicrobial agents preferably, non-synthetic agents, to combat infections. In 2017, WHO cries out for an emergency aid in research and development towards a world's health immediate need as there is an alarming decline rate of antibiotics particularly, the ESKAPE pathogens. If care is not taken in this century, the infections caused by these pathogens may return the world back to orthodox ages (WHO 2017). Hence the need to investigate the antibacterial properties of bacteria isolated from fish mucus.

Materials And Methods

Mucus collection and preparation for antibacterial activities

Five (5) healthy live samples each of two different fishes (*Chrysichthys nigrodigitatus and Solea solea*) regardless of age and sex were purchased from riverine area in badagry, Lagos, Nigeria. The fish samples were collected into sterile perforated container filled with water and labeled accordingly. Mucus collection was done by starving the fishes for one day without prior application of anesthesia and transported to the laboratory for microbiological analysis observing the modified method of SUBRAMANIAN et al. (2007). Briefly, Whatman no 1 filter paper was used to remove the mucus from the fishes by gently moving it from the head of the fish to the tail in order to slough off the fish mucus. This was done separately for each fish specie. Spam and intestinal cross-contamination were prevented by avoiding mucus collection through the ventral side. The aseptically collected mucus sample were mixed well with equal quantity of sterilized physiological saline (0.85% NaCl) in readiness for microbial isolation and some parts of the mucus was centrifuged at 5,000 rpm for 15 mins. The supernatant was placed in vials and kept at 4°C for future antimicrobial studies.

Acquisition and conservation of pathogenic bacterial strains

The pathogenic bacterial strains were obtained from the Federal Institute of Industrial Research (FIIRO), Lagos, Nigeria. Antibacterial activities of fish skin mucus extracts was tested against four pathogenic bacteria which includes *Escherichia coli, (*ATCC 25922*), Staphylococcus aureus,* (ATCC 25923*) and Bacillus subtilis (ATCC 6633)* and a common gram negative fish pathogenic bacteria *Aeromonas hydrophilla (ATCC 35654).* All the bacterial strains were maintained by growth in nutrient broth observing a standard biomedical safety procedures and conditions. A 10 ml of prepared nutrient broth was poured in a flask and one loop of each targeted test bacteria was added to the flask and incubated at 37°C for 24 hrs.

Isolation and characterization of associated bacteria

The method of isolation was by culture method. This was done by preparing media such as Nutrient agar (Oxoid, UK), MacConkey agar (Oxoid, UK) and Mueller Hinton agar (Oxoid, UK) following manufactures' specification. This was followed by aseptically inoculating the serially diluted mucus on the media plate of Nutrient agar (Oxoid, UK), blood agar (Oxoid,

UK), mannitol salt agar (Oxoid, UK) and MacConkey agar (Oxoid, UK). The inoculated plates were labeled appropriately and incubated at a room temperature of 37°C for 24 hrs. The microbial isolates were sub cultured until pure culture were obtained before transferring to an agar slant for further use. The associated bacteria isolates were identified morphologically (size, form, colour, consistency, edges, elevation and opacity). They were further identified using Gram staining. Biochemical characterization such as indole, oxidase, voges proskauer, triple sugar iron, urease, hydrogen sulfide production test for solubilizing bacteria citrate, catalase and motility test following Bergey's manual of systematic bacteriology (HOLT et al. 1994).

Antibiotics sensitivity test

Antibiotic susceptibility test was achieved by Kirby Bauer Disc diffusion susceptibility method according to Clinical and laboratory standard institute (CLSI 2009). A Mueller-Hinton agar plate was prepared according to manufacture^{r}"s specification. Three to five well-isolated colonies of the test bacteria were selected from an agar plate and suspended in 5 ml of nutrient broth. The turbidity of the broth culture was adjusted to 0.5 McFarland standard. Standardized inoculums of the organism were swabbed on the entire Mueller-Hinton agar plate within 15 minutes. At about three minutes thereafter, paper discs impregnated with the following antimicrobial agents; Augmentin (AUG) 30 µg, Chloramphenicol (CH) 30 µg, Septrin (SXT), 30 µg, Gentamycin (CN) 10 µg, Streptomycin (STR) 30 µg, Amoxacillin (AMX) 30 µg, Sparfloxacillin (SP) 30 µg, Ciprofloxacin (CPX) 30 µg, Pefloxacin (PEF) 30 µg, Tarivid (OFX) 10 µg (all from Oxiod UK) were positioned on the agar plate using sterile forceps. These plates were left for about 30 mins for predifussion to take place before incubation. The plates were incubated at 37°C for 24 hours. The diameter of zone of inhibition was measured to nearest millimeter.

Screening for antibacterial activity of organisms isolated from fish mucus

The bacterial isolates from the fish mucus were tested against pathogenic bacterial strains for their antibacterial potency using the spot on lawn assay and disc diffusion method.

The spot-on lawn antimicrobial assay used had little modification. Briefly, Mueller Hinton agar was prepared and at molten state around 40°C, the pathogenic organisms were evenly mixed with the agar and poured into the plate. Varied aliquots diluent of the test organisms are

dripped onto the media that has been seeded with the pathogenic microorganisms. After aerobic incubation at 37°C for 24 hours, the antimicrobial activity was measured and expressed as inhibition zone diameter.

In disc diffusion assay, discs of 6 mm were absorbed with supernatant and placed on the agar seeded with pathogenic bacteria strains. After overnight incubation at 37°C, the inhibition zone is evaluated in millimeter (mm) based on the clear zone around the paper disc according to SOOMRO et al. (2007).

Data analysis

The data obtained were first subjected to descriptive statistics (mean and standard error of the mean). Analysis of Variance (ANOVA) was used to determine significant differences in mean across all the groups. *P*-values of less than 0.05 were considered as significant using SPSS software.

Result and discussion

Study in this research generates an insight into the antibacterial activities and antibiotics susceptibility pattern of bacteria isolated from fish mucus on clinical isolates using spot on lawn and disc diffusion method. In this case, a total of 30 bacterial isolates were identified; *Salmonella typhi* (40%), *Proteus mirabilis* (8%), *Klebsiella pneumoniae* (20%), *Shigella sonnei* (20%), and *Citrobacter freundii* (12%) – Figure 1. The bacteria identified through biochemical tests are *Klebsiella pneumoniae, Salmonella typhi, Proteus mirabilis, Shigella sonnei, Citrobacter freundii* (Table 1).

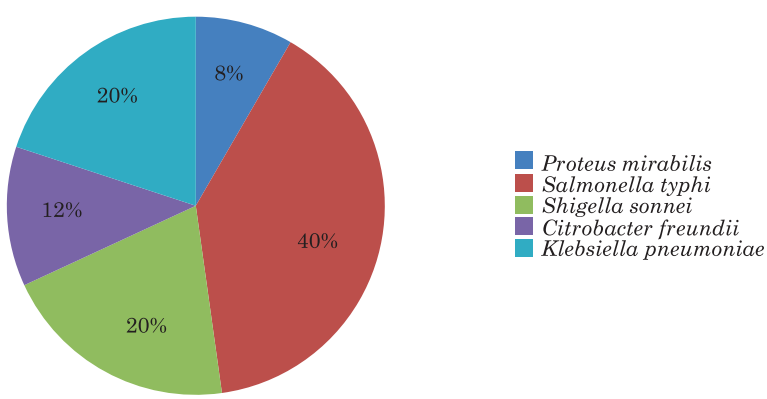

Fig. 1. Percentage occurrence of bacteria isolated from fish mucus

Table 1

Biochemical identification of bacteria isolated from fish mucus

S/N	ORG	GR	IN	OX	MO	CA	UR	CI	H_2S	TSI		
										SU	LA	GL
1.	*Klebsiella pneumoniae*	–	–	–	+	+	+	+	–	+	+	+
2.	*Proteus mirabilis*	–	–	–	–	+	+	+	+	–	–	+
3.	*Shigella sonnei*	–	+	–	–	+	–	–	–	–	–	+
4.	*Citrobacter freundii*	–	–	–	+	+	–	+	+	–	–	+
5.	*Salmonella typhi*	–	–	–	+	+	–	–	+	–	–	+

Explanations: ORG – organism, GR – gram reaction, IN – indole, OX – oxidase, CA – catalase, UR – urease, CI – citrate utization, GL – glucose, H_2S – hydrogen sulphide, TSI – triple sugar iron, SU – sucrose, LA – lactose

The fact that, *Salmonella typhi* was the most prevalent bacterial specie contradict the report of MANHONDO et al. (2018) who isolated a different set of bacteria sp. from fish in Zimbabwe. In the same vein, our results also contradicts that of HAFSAT et al. (2015) who reported *Staphylococcus aureus, Streptococcus* spp. *and Escherichia coli* as the most frequent bacterial species in the epidermal layer of fish. The difference in the bacteria species recorded may be due to seasonal variation, geographical location as well as sources of indiscriminate disposal of refuse in water bodies (CABRAL 2010).

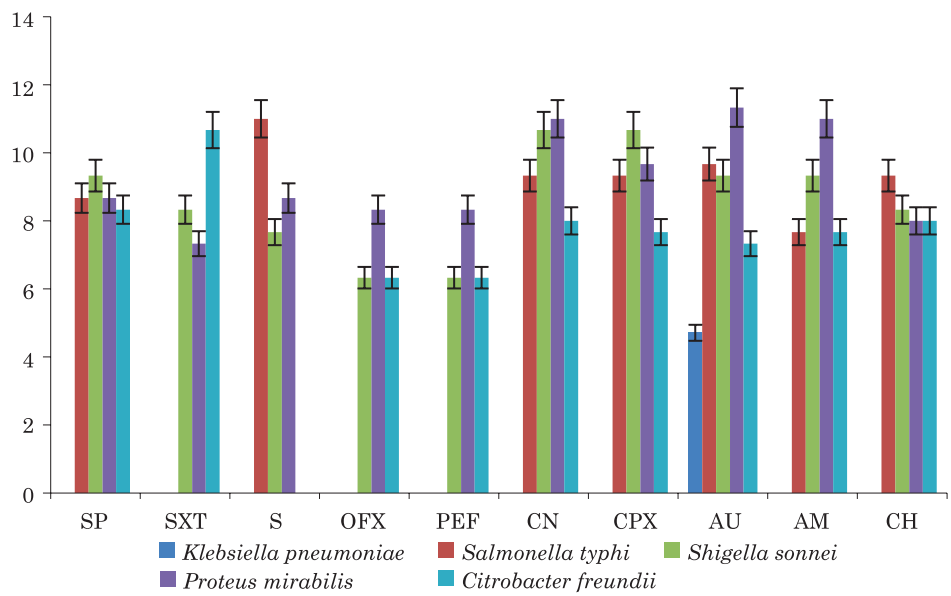

Fig. 2. Graphical representation of antibacterial activity of selected antibiotics on fish mucus isolates: SP (Sparfloxacin 10 µg), SXT (Septrin 30 µg), S (Streptomycin 30 µg) OFX (Tarivid 10 µg), PEF (Pefloxacin 30 µg), CN (Gentamycin 30 µg), CPX (Ciprofloxacin 30 µg), AU (Augmentin 10 µg), AM (Amoxacilllin 30 µg), CH (Chloramphenicol 30 µg)

The antibiotics sensitivity test of the fish mucus isolates as depicted in Figure 2 revealed that *Klebsiella pneumoniae* is resistance to virtually all the ten different antibiotics used except Augmentin (4.71±1.21). In contrast, *S. typhi* and *S. sonnei* were susceptible to all antibiotics used while *P. mirabilis* is susceptible to most of the antibiotics but resistant to three (pefloxacin, septrin and arivid). Furthermore, *C. freundii* showed resistance to only Streptomycin while being susceptible to the rest of the antibiotics. This result corroborates with the study of MANHONDO et al. (2018). They identified bacteria that were multi resistant to different antibiotics used with the exemption of *P. mirabilis*. Non-susceptibility of some of the identified isolates to antibiotics may be attributed to the presence of non-biodegradable metal disposal into the water bodies, hence, accelerating the natural selection in water bodies, enhancing the movement of genes coding for resistance of antibiotics among water loving bacteria (ALANIS 2005, SEILER and BERENDONK 2012).The antibacterial activities of fish mucus isolate *P. mirabilis* against the clinical isolates using overlay antimicrobial assay inhibited *E. coli*, *B. subtilis* and *A. hydrophila* with a zone of inhibition 2.67±1.15 mm, 4.00±1.73 mm and 1.00±1.00 mm respectively. In the disc diffusion method, *P. mirabilis* inhibited *E. coli* (3.00±1.48 mm), *B. subtilis* (5.00±2.31 mm), *A. hydrophila* (4.00±2.11 mm) – Figure 3. This result is in

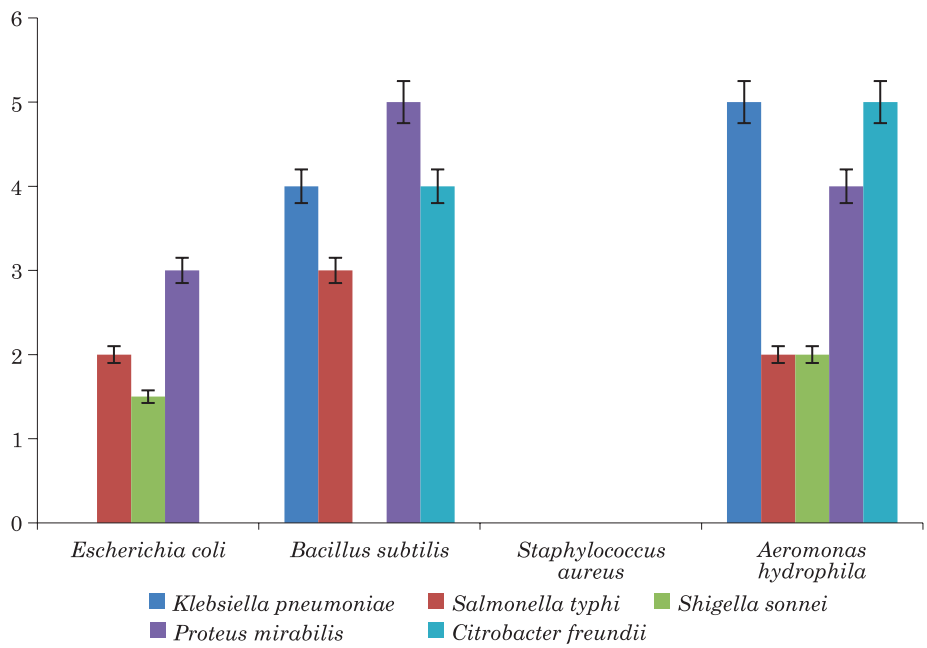

Fig. 3. Graphical representation of antibacterial activity of fish mucus isolates on clinical isolates using spot on lawn/overlay assay

agreement with the study of NAZ and RASOOL, (2013), who reported that *P. mirabilis* produces antibacterial proteins that are capable of inhibiting other organisms. *C. freundii* using spot on lawn inhibited *E. coli* (3.67±0.58 mm) and *B. subtilis* (3.33±1.53 mm). However, it showed no significant effect on *Staph. aureus* (0.00±0.00mm) and *A. hydrophila* (0.00±0.00 mm) in the overlay antimicrobial assay.

Contrastingly, *C. freundi* have no antibacterial effect on *E. coli* and *B. subtilis* but it inhibited *B. subtilis* (4.00±2.74 mm) and *A. hydrophila* (5.00±2.61 mm) in the disc diffusion assay (Figure 4). Our finding is in line with the study of ROBERT et al. (2012), who reported the production of antimicrobial compound by *C. freundii*. In this study, *Klebsiella pneumoniae* antimicrobial assay using overlay method showed inhibition on *B. subtilis* (3.67±2.8 mm), *S. aureus* (0.67±0.58 mm) and *A. hydrophila* (2.33±1.15 mm) while only *B. subtilis* (4.00±2.11 mm) and *A. hydrophila* (5.00±2.31 mm) were inhibited when disc diffusion method is used.

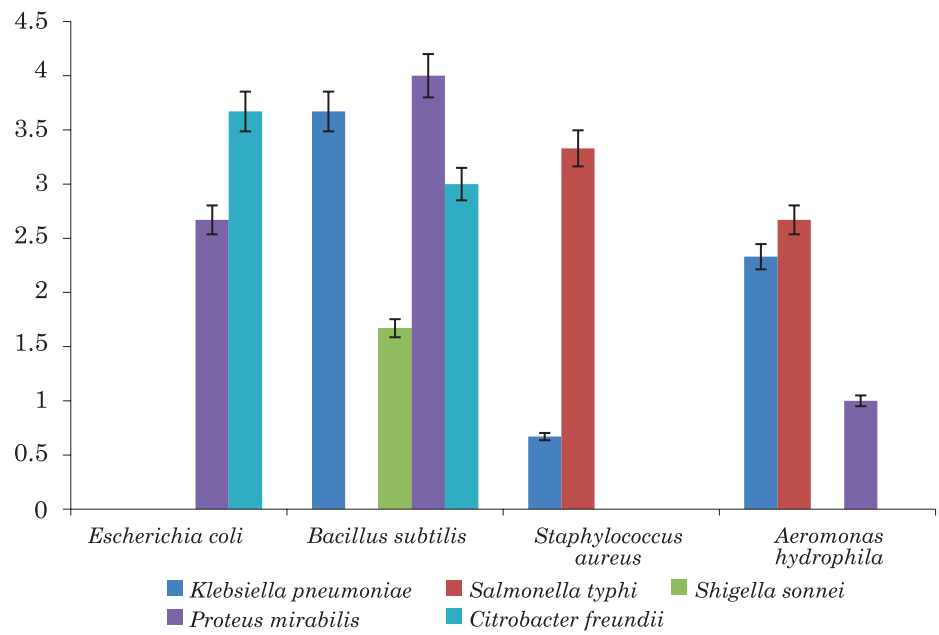

Fig. 4. Graphical representation of antibacterial activity of fish mucus isolates on clinical isolates using disc diffusion method

S. typhi inhibited only *S. aureus* (3.33±0.58 mm) and *A. hydrophila* (2.67±1.151 mm) using overlay assay, but inhibited more pathogenic organisms (*E. coli* (2.00±1.30 mm), *B. subtilis* (3.00±1.31 mm), *A. hydrophila* (2.00±1.34 mm)) when disc diffusion method was employed. *S. sonnei* inhibited only *B. subitilis* (1.67±2.08 mm) when overlay assay was used.

However, it inhibited *E. coli* (1.50±0.54 mm) and *A. hydrophila* (7.00±0.21) using disc diffusion method (Figure 4). This implies that *S. sonnei* demonstrated the least antimicrobial effect among the fish mucus isolates while *P. mirabilis* demonstrated the highest antibacterial effect.

In contrast*, Staph. aureus* showed a very high resistance to the antibacterial activity of fish mucus isolates. The inhibitory effects of the organisms isolated from fish mucus as reported in this study is in conformity with previous reports (MIDHUN et al. 2017a,b). The inhibitory action exhibited by the bacteria spp. were attributed to mass or holistic secretion of antagonistic materials which include bacteriocins, siderophores, antibiotics (SEBASTIAN et al. 2018), and lysosomal enzymes (RAY et al. 2012).

Previous studies have also indicated that, environmental isolates of *Bacillus* spp. produced bacteriocin like peptides such as lichenicidin and lantibiotic, which gave 100% resistant to the growth of both gram-negative and gram-positive bacteria (MUKHERJEE et al. 2016, SEBASTIAN et al. 2018). Similarly, another research conducted by SEBASTIAN et al. (2018) on inhibitory properties of *Bacillus coagulans* found in gut of fishes against pathogens reported that *B. coagulans* may protect the fish from invading pathogenic bacterial cells. (SEBASTIAN et al. 2018). This study further agrees with the conclusion of a study carried out in corals on antibiotic activity in fish which stated that close to a quarter of bacteria cultured from the mucus of the elkhorn coral, *Acropora palmata*, exhibited antagonistic properties against a range of pathogenic test strains whereby 8% were directly active against a causative agent of a disease in the particular species (RITCHIE 2006).

Conclusion

These results confirm that the protection found in fish against surrounding intruders could have been acquired from their epidermal slime layers. Hence, harnessing fish mucus for its antibacterial properties will not be out of place. Major limitation to the study is the fact that only two species of fishes were used in this study, hence we can't generalize antibacterial potential of fish mucus.

Translated by dr. Ojo Abidemi

Accepted for print 19.08.2021

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