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Antibacterial and Synergistic Potential of Scale Extracts from *Oreochromis mossambicus* against Bacterial Pathogens

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SUMMARY

There is a severe concern about evolving transmittable infections and the increasing drug resistance in bacterial pathogens. It is the role of pathogenic bacteria to cause wide variety of deep-rooted infections. Tilapia (*Oreochromis mossambicus*) belongs to the family cichlidae. Fish relies on their instinctive immune mechanisms to combat a wide range of microorganisms. Less work was done on the use of animals for the treatment of different infectious diseases. Fish scales are solid waste discarded by humans. In current work *Oreochromis mossambicus* scales were subjected for antibacterial activity, synergistic activity and antibiofilm activity. The antimicrobial activity and was carried out by Agar well diffusion method and sensitivity or synergistic test was performed via Disc diffusion method against *Staphylococcus aureus, Proteus mirabilis, Klebsiella pneumonia* and *Pseudomonas aeruginosa* bacteria. Results showed that the potential antibacterial activity against tested pathogens, combined effect of fish scale extract (OM-Aqu) and antibiotics were efficient against the four tested bacterial strains as compared to OM-Met and OM-E. All the tested pathogens were highly sensitive against the (OM-Aqu) and antibiotic. Antibiofilm activity was in accordance with antibacterial activity. This study concluded, that biological waste material like fish scales can be used for medical purposes the prepared extract will be use to minimize or eradicate biofilm related infections and could be use in drugs.

Keywords: *Oreochromis mossambicus*, Antibacterial effect, Crystal violet assay, Synergistic effect.

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INTRODUCTION

A pathogenic bacterium is generally classified as "any bacterium that has the capability to cause disease and its aptitude to cause disease is termed as pathogenicity (Balloux and van Dorp, 2017). Anti-microbial resistance are of serious consideration to developed regions because of the over use of antimicrobial agents, inadequate steps to manage infections, and extensive supply of counterfeit or under-standard drugs (Mendelson and Matsoso, 2015). The prevalence of anti-microbial resistance is presently most noteworthy public health concerns of people and (anti-biotic resistance genes) are regarded the environment's main pollutant (Sabri et al., 2020).

In the age of expanding information, the resurgence of traditional therapeutic approaches has become quite a story in recent years. Surprisingly, almost 80% of

individuals worldwide now turn to traditional treatments for their primary health needs. They appreciate these cures because they are inexpensive and effective, with little to no side effects. Among these restorative methods is something known as zootherapy. This refers to the use of medications derived from animals or their products, and it is increasing popularity (Amjad et al., 2020; Ahmad et al., 2021; Altaf et al., 2021; Bashir et al., 2021; Hamid et al., 2021; Saleem et al., 2021; Rahman et al., 2022; Saeed et al., 2022; Iqbal et al., 2023). Animals used as traditional medicines among several other therapies practiced globally, the zootherapy is an important and significant option in modern society (Alves et al., 2012).

Oreochromis is a prominent species of fish found in tropical freshwater, particularly useful for fish farming, and it has been managed through various introductions and movements. According to scientists, some *Oreochromis* species, such as Nile tilapia *(O. niloticus),* are excellent for fish farming (Diedericks et al., 2021). Despite the importance of this fish, literatures on the antibacterial properties of the fishes are limited whereas lack of work is on fish scale extract which are discarded as solid waste biological material. Hence, this present study was undertaken in an attempt to investigate and assess the antibacterial activity and synergistic activity of the epidermal mucus of *Oreochromis mossambicus*. This will provide a good understanding of the antibacterial compounds in the scales extract.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

All the chemicals and solvents used in this experiment were analytical grade with ≥ 95% purity were obtained from CARLO ERBA (Itlay), Bioworld (USA), Bioanalyse Turkey and Sigma Aldrich (Germany). Apparatus used were Weighing balance (SHIMADZU Japan), Autoclave (Sturdy Apex Taiwan), thermostat incubator with shaker (ZHP-100 China), Hot plate and centrifuge (SCILOGEX China), UV-Vis Spectrophotometer (Pg instruments USA), Laminar Flow (ESCO world class Singapore), Micropipettes (Milward UK).

COLLECTION AND IDENTIFICATION OF SAMPLE

Tilapia (*Oreochromis mossambicus*) was gained from Gujranwala district, the city of Pakistan. The fish sample was identified by expert, Zoology Department, Faculty of Science, "Women University of Azad Jammu and Kashmir, Bagh, Pakistan".

PREPARATION OF EXTRACT

The scales were washed with distilled water for removing particles of dust and dried in oven at (45 ºC ± 2) temperature. The dried parts were chopped into powder form. Three extracts (Aqueous (OM-Aqu), Methanolic (OM-Met) and ethanolic (OM-E)) were prepared by applying process after slight modifications. By using the pestle and mortar the fish sample was pulverized finely. The 100mL extract was prepared using distilled - water double. Filtration of prepared extract was done by using Whatman N0. 1 filter paper to gain perfect extract.

Bacterial pathogens

The antibacterial activity of was assessed against three Gram negative (*Klebsiella pneumonia, Pseudomonas aeruginosa,* and *Proteus mirabilis*) and one Gram positive (*Streptococcus pyogenes*) bacterial pathogens, isolated from various clinical samples.

Agar well diffusion method

The Agar Well Diffusion method is determining the antibacterial activity of the compounds (Figure 1). A microbial solution was applied to the surface of an agar plate during this procedure. Following that, three 5 mm diameter wells were created with sterile yellow tips, and agar plugs were carefully retrieved with sterile needles. Following that, roughly 25 l of each OM-Aqu, OM-Met, and OM-E solution (at 0.1 mg/ml) were deposited into the prepared wells, along with a control solvent sample (DMSO). After that, the plates were placed in a 37°C oven for 24 hours. DMSO was used as a negative control, whereas Chloramphenicol discs (10 g/ml) were used as a positive control. Following methodology (Seeley Jr and VanDemark, 1962), the zones where bacterial growth was prevented were measured in millimetres after a 24-hour period. According to Hammer et al. (1999), the diameter of clean regions around each well was measured using a scale. The area of inhibition was used to categorise the level of growth inhibition: 0 mm designated insensitivity, 1-5 mm denoted low sensitivity, >5-8 mm denoted moderate sensitivity, and >8-10 mm denoted high sensitivity.

Sensitivity Test

The agar disc diffusion method was used to assess antibiotic sensitivity (Figure 2), including ciprofloxacin (10 g) and chloramphenicol (10 g), against the bacterial strains under investigation. Following the approach indicated by Sharma et al. (2012), these antibiotics acted as a positive control. Only the OM-Aqu extract was utilised in conjunction with the antibiotics in this investigation. A combination effect was examined in this experimental setup. To do this, antibiotic discs containing ciprofloxacin (10 g) and chloramphenicol were infused with each extract and then dried briefly under a laminar flow. Following that, three discs infused with each extract were placed at equal intervals on the agar's surface. This was accomplished by carefully positioning each antibacterial disc using sterilised forceps. Petri plates were placed in incubator at 35°C for 24 hours. After incubation, the zones of inhibition were calculated and compared (Verma and Mehata, 2016).

Anti-biofilm Assay

The anti-biofilm activity was evaluated using the Crystal Violet assay with minimal modifications, as described by O'Toole (2011). The bacteria were grown overnight at 37°C in Borosilicate tubes (Minitek, USA) containing 3 ml of nutritional broth medium with a concentration of 25 mg/ml of OM-Aqu, OM-Met, and OM-E. The positive control was chloramphenicol, while the negative control tubes contained simply nutritional broth. The broth medium was decanted after incubation, and adhering cells were stained with 120 l of a 0.1% crystal violet solution. The Borosilicate tubes were then incubated at room temperature for 10-20 minutes before being washed with water to remove excess unattached

cells and dye. Following staining, 30% acetic acid was added to dissolve the crystal violet, and the mixture was incubated at room temperature for 10-20 minutes. A spectrophotometer was used to measure the solubilized crystal violet at 550 nm, with a blank reference of 30% acetic acid in water.

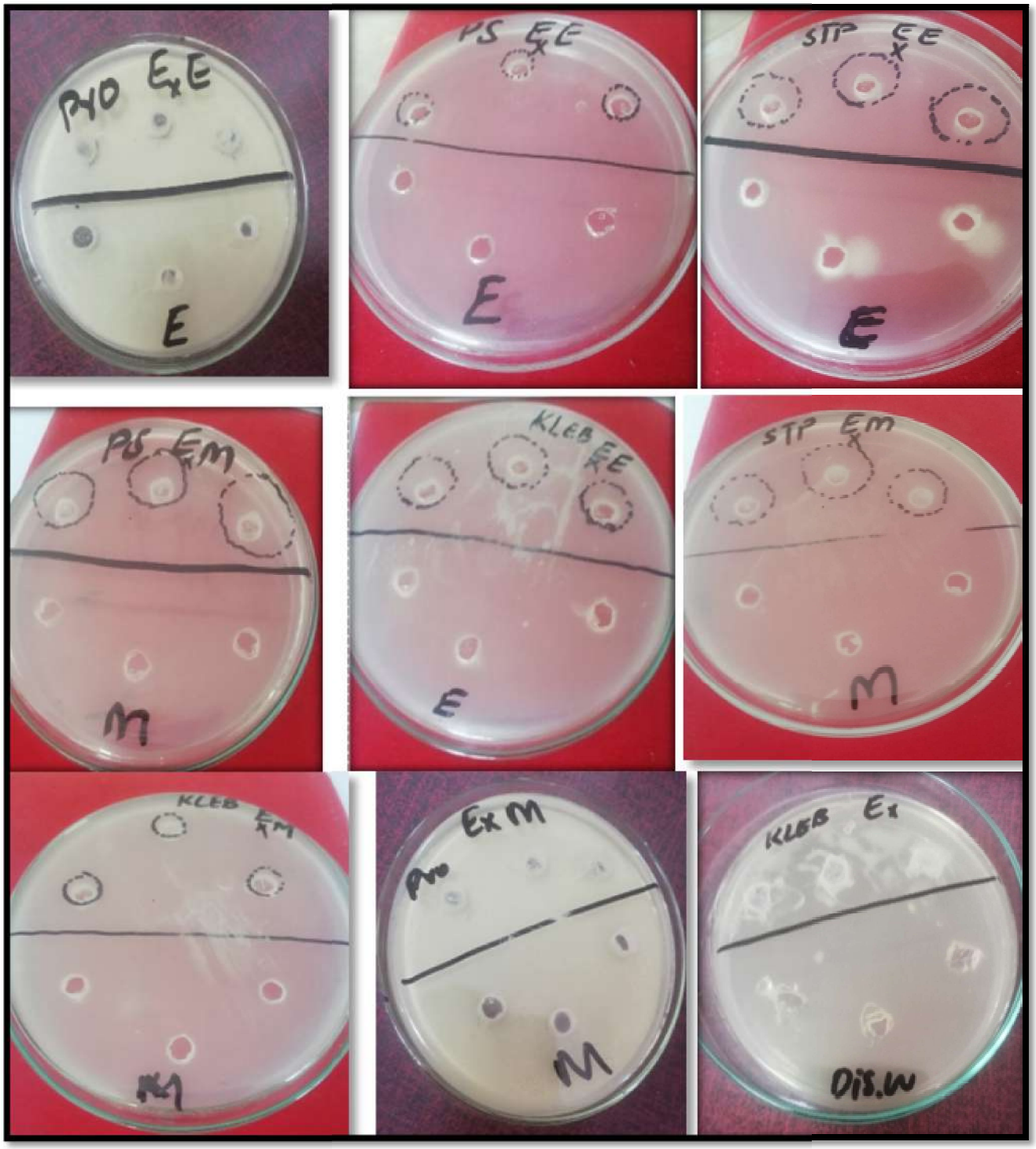

Figure 1: Antibacterial activity of OM-Aqu, OM-E and OM-Met against *Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus mirabilis* and *Streptococcus pyogene.*

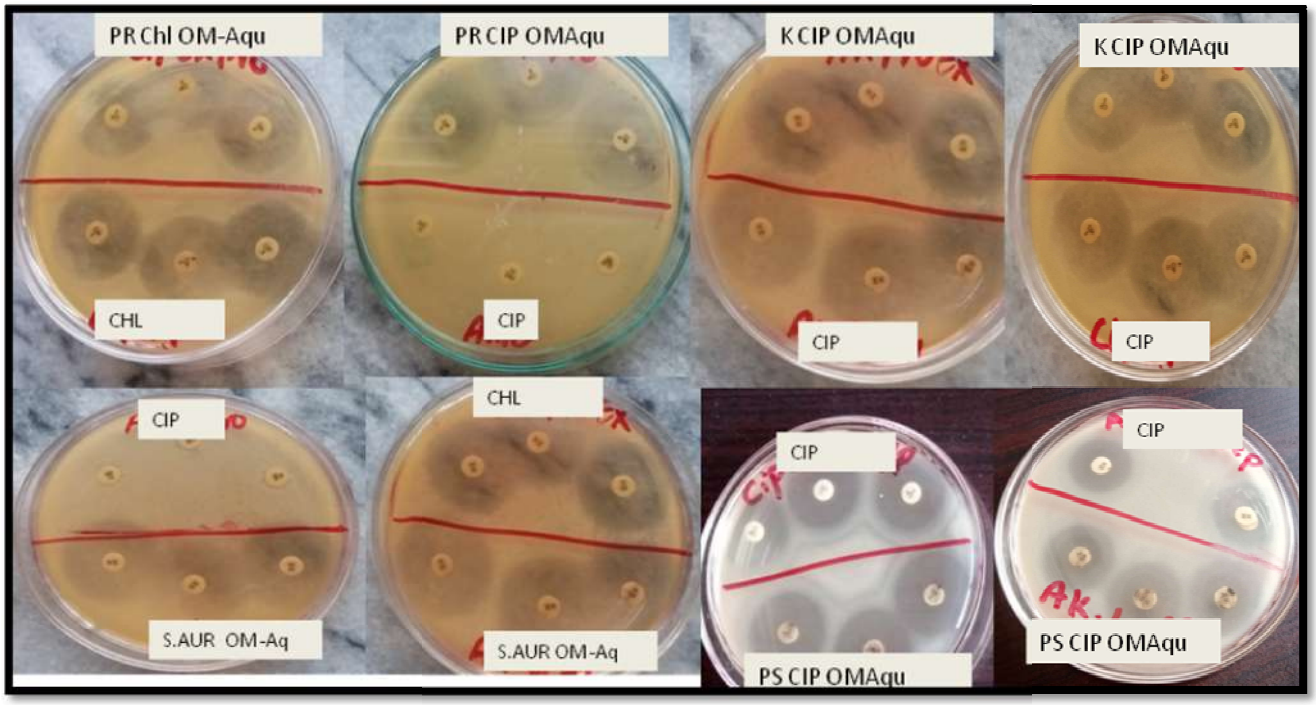

Figure 2: Sensitivity testing of antibiotics and combined effect of extracts along with Chloremphenicol, Ciprofloxacin against *Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus mirabilis* and *Streptococcus pyogenes.*

RESULTS AND DISCUSSION

During the antibacterial tests, it was discovered that OM-Aqu had a remarkably high toxicity against the identified pathogenic bacteria when compared to the other extracts. Furthermore, at 0.10 g/mL concentrations, OM-Aqu, OM-Met, and Omet demonstrated significant antibacterial efficacy. Maximum zone of inhibition was noted at 0.10 g/mL concentration of OM-Aqu against *Proteus mirabilis* (14±0 mm), where as significant effect was shown against *Pseudomonas aeruginosa* (11±2.3mm), *Klebsiella. pneumoniae* (10±6.4 mm), *Staphylococcus. aureus* (10.0±2.0 mm). Similarly OM-Met and OM-Met showed minimum zones against all the tested bacteria. DMSO taken as negative control showed no zones of inhibition where as chloramphenicol taken as positive control showed maximum zones of inhibitions (Table 1).

In the proposed work, the effect of OMAqu, OMmet and OMet on the formation of biofilm was examined by crystal violet assay. The findings showed that, the presence of OMAqu, OMmet, OMet and chloramphenicol reduced the biofilm formation.The results showed that among the three extracts (OMAqu,OMmet, OMet) the OMAqu seemed to have the highest ability to minimize biofilm formation at a significant range. The antibiofilm results of OMAqu were inconsistence with antibacterial results of OMAqu (Table 3).

Sensitivity test and the synergistic effect of OM-Aqu extract was further done against tested bacterial pathogens because OM-Aqu showed strong inhibition at the concentration of 0.10 g/mL. The effect of chloramphenicol against *Klebsiella pneumonia* (21.3±3.2), and *Proteus mirabilis* (25±5) as effective as compared to

*Pseudomonas aeruginosa*w (17.3±0.5) and *Staphylococcus aureus* (15±0*).* The effect of methanolic and ethanolic extracts was not much effective against the bacterial strains as compare to the chloramphenicol, so only OM-Aqu extract was used in combination with antibiotics (table 2).

The combined effect of OMAqu+antibiotics (ciprofloxacin (CIP) and chloremphenicol (CH)) was further done through agar disc diffusion assay against only four pathogenic bacteria as these pathogens are highly sensitive to OMAqu. The range of inhibition zone was recorded between 15 ± 0 to 33±3.6. This showed that the aqueous extract was most significant along with antibiotics in contrast to chloramphenicol (CH). Satisfactory synirgestic effect of OMAqu+CH and OMAqu+CIP has been detected incontrast to all verified pathogenic microbes; *Sataphilococus aurious, Proteus mirabilus, Kalebsillae pnwmunia,* and *Peseudomonhas aurginosa* whereas the minimum effect of CH and CIP was examined against all tested pathogens; *Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumonia.*

The significant inhibitory zones of OMAqu+CH against all four tested pathogens were recorded as; *Staphylococcus aureus* (41.6 ±25.1), *Proteus mirabilis*(39±3.6), *Klebsiella pneumonia*(38±1),and *Pseudomonas aeruginosa* (37.3±0.5) whereas the minimum inhibition zones of individual CH were recorded as; *Pseudomonas aeruginosa* (17.3±0.5), *Staphylococcus aureus* (15±0), *Proteus mirabilus (*25±5), *Klebsiella pneumonia* (21.3±3.2) respectively.

The effective inhibition zones of OMAqu+CIP against four pathogens were observed as; *Klebsiellapneumonia* (43±2.6), *Pseudomonas aeruginosa* (37.3±0.5), *Proteusmirabilis* (35±0), and *Staphylococcus aureus* (15±0) whereas the lowest effect of only CIP against the tested strains were minimum and zones of inhibition were measured as; *Klebsiella pneumonia (*28.3 ± 1.5*), Staphylococcus aureus* (0), *Proteus mirabilis* (0)*, Pseudomonas aeruginosa* (17.3±0.5) respectively. Results showed that the combined effect of fish extract (OMAqu) and antibiotics was efficient against the four tested bacterial strains. All the tested pathogens were highly sensitive against the (OMAqua) and antibiotic. The maximum inhibition zone of OMAqu+CH was measured against *Staphylococcus aureus* (33±25.1). The highest inhibitory effect of OMAqu+CIP was measured against *Klebsiella pneumonia* (33±2.6) respectively.

Table 1: Antibacterial activity of fish extract against *Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus mirabilis* and *Streptococcus pyogenes*

Tested sample pathogens	OM-Aqu	OM-Met	OM-E	Chloramphenicol (10ug)
Klebsiella pneumonia	8.6±6.4	2±0	7±1	21.3±3.21
Pseudomonas aeruginosa	4.3±2.3	8.6±2.8	3±0	17.3±0.57
Staphylococcus aureus	10±2	9±1	7.6±o.5	2±0
Proteus mirabilis	6±0	7.6±0.5	5±0	25±5

The bacterial growth sensitivity was linked as; 0.0 indicated no sensitivity, (15-25) showed maximum sensitivity, (8-16) specified intermediate sensitivity, lowest sensitivity below 7.

Table 2: Synergistic effect of OMAqu along with standard antibiotics against bacterial pathogens (*Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus mirabilis* and *Streptococcus pyogenes*).

Bacterial pathogens Treatments	Inhibition zone (mm)			
	Klebsiella pneumonia	***Proteus mirabilis***	***Staphylococcus aureus***	***Pseudomonas aeruginosa***
OM-Aqu+CH	33±1	33±3.6	33±25.1	33.3±0.5
CH	21.3±3.2	25±5	15±0	17.3±0.5
OM-Aqu+CIP	33±2.6	32±0	15±0	33.3±0.5
CIP	28.3±1.5	0	0	17.3±0.5

The bacterial growth sensitivity was linked as 0.0 indicated no sensitivity, (25-33) represented maximum sensitivity, (15-25) indicated intermediate sensitivity, lowest sensitivity below 15. CH (Chloremphenicol), CIP (Ciprofloxin).

Table 3: Antibiofilm activity of OMAqu, OMmet, Omet extracts from fish scales against tested bacterial pathogens.

Test organisms	OM-Aqu	OM-Met	OM-E	Pathogen	Chloramphenicol (CH)
Klebsiella pneumonia	0.082	0.034	0.457	1.41	0.027
Pseudomonas aeruginosa	0.062	0.026	0.053	1.25	0.023
Staphylococcus aureus	0.033	0.166	0.023	1.61	1.21
Proteus mirabilis	0.023	0.167	0.117	1.033	1.430

Certain bacterial germs have developed resistance to routinely used antibiotics, surviving and even multiplying in the presence of these drugs. Furthermore, the formation of biofilms is inextricably tied to the problem of antibiotic resistance (Lynch et al., 2007).

The possibility of discovering previously identified metabolites in marine organisms is 500 times greater than in land-dwelling species. The marine environment's biochemical resources represent a huge reservoir of prospective medicinal and commercial goods. This emphasises the importance of bioprospecting research in the search for novel medicinal agents, such as antibacterial and anti-proliferative chemicals (da Cunha et al., 2013). Furthermore, as highlighted by Marston et al. (2016), the growing issue of antimicrobial resistance poses a huge threat to global well-being while also contributing considerably to the global rise in healthcare expenses. This emphasises the urgent need to discover novel antibacterial chemicals. This existing work has been accompanied to determine the presence of defensive antibacterial potency in *O.mossambicus* scale extracts. The fish extract (OMAqu) was tested for bactericidal potency contrast to four pathogenic bacteria by agar well diffusion assay. Three solvent extracts were used methanolic, ethanolic and aqueous extract of OM. All extracts showed effective inhibition against tested bacterial pathogens. Fish skin mucus is important in the innate immune system, acting as the first line of defence against infections. While various studies have investigated and confirmed the antibacterial properties of fish skin mucus, the majority of these studies focused on assessing its efficacy against marine microbial strains, with potential uses in aquaculture, as demonstrated by Tiralongo et al. (2020).

There is lack of work on fish scales so the current result revealed that the aqueous extract (OMAqu) was more active against all four tested bacterial pathogens. Results showed that the combined effect of fish extract (OMAqu) and antibiotics was efficient against the four tested bacterial strains. All the tested pathogens were highly sensitive against the (OMAqua) and antibiotic. The maximum inhibition zone of OMAqu+CH was measured against *Staphylococcus aureus*(33±25.1). The highest inhibitory effect of OMAqu+CIP was measured against *Klebsiella pneumonia* (33±2.6) respectively.

Despite the fact that a handful of medications have been developed from marine invertebrates, the use of marine vertebrates as prospective sources for innovative therapeutic agents has been very limited in the marine environment (Luer and Walsh, 2018). Interestingly, discarded fish have unrealized potential as a handy and cost-effective resource for isolating novel physiologically active chemicals (Hellio et al., 2002). In this regard, fishes' considerable and well-documented antibacterial characteristics make them excellent candidates for discovering new antimicrobial compounds with potential advantages for human health. Fish scales, which are typically disposed as solid trash, are an underutilised resource in this regard. Surprisingly, fish scales' antibacterial potential has largely gone untapped. The new study shows that fish scales, which are regularly thrown from fisheries, could indeed be a relevant, conveniently accessible, and cost-effective source for identifying novel biologically active chemicals.

CONCLUSION

The extract from *O. mossambicus* scales shown significant antibacterial effectiveness against both Gram-negative and Gram-positive bacterial infections. This shows that these scale extracts could be attractive candidates for the development of antibacterial medicines, especially against bacterium strains resistant to numerous treatments. Fish scales have potential applications in a variety of disciplines, including antibacterial systems. In conclusion, it is clear that continuous study and endeavours to delve into and exploit the biological features inherent in the readily available and cost-effective resource of fish scales are required.

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