



# Histone acetyltransferases derived RW20 protects and promotes rapid clearance of *Pseudomonas aeruginosa* in zebrafish larvae

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## Abstract

*Pseudomonas* is a group of bacteria that can cause a wide range of infections, particularly in people with weakened immune systems, such as those with cystic fibrosis or who are hospitalized. It can also cause infections in the skin and soft tissue, including cellulitis, abscesses and wound infections. Antimicrobial peptides (AMPS) are the alternative strategy due to their broad spectrum of activity and act as effective treatment against multi-drug resistance pathogens. In this study, we have used an AMP, RW20 (<sup>1</sup>RPVKRKKGWPKGVKRGPPKW<sup>20</sup>). RW20 peptide is derived from the histone acetyltransferases (HATs) of the freshwater teleost, *Channa striatus*. The antimicrobial prediction tool has been utilized to identify the RW20 sequence from the HATs sequence. We synthesized the peptide to explore its mechanism of action. In an *in vitro* assay, RW20 was challenged against *P. aeruginosa* and we showed that RW20 displayed antibacterial properties and damaged the cell membrane. The mechanism of action of RW20 against *P. aeruginosa* has been established *via* field emission scanning electron microscopy (FESEM) as well as fluorescence assisted cell sorter (FACS) analysis. Both these experiments established that RW20 caused bacterial membrane disruption and cell death. Moreover, the impact of RW20, *in-vivo*, was tested against *P. aeruginosa*-infected zebrafish larvae. In the infected larvae, RW20 showed protective effect against *P. aeruginosa* by increasing the larval antioxidant enzymes, reducing the excess oxidative stress and apoptosis. Thus, it is possible that HATs-derived RW20 can be an efficient antimicrobial molecule against *P. aeruginosa*.

**Keywords** *Pseudomonas aeruginosa* · Histone acetyltransferases · Antimicrobial peptide · Zebrafish · Apoptosis

## Introduction

*Pseudomonas aeruginosa* is a common pathogen in the healthcare-associated infections, especially in critically ill or immunocompromised patients. It is a versatile pathogen that can cause potent infections (Hirsch and Tam 2010). It is responsible for 10% to 20% of all the infections in most hospitals. Patients who are at risk of *Pseudomonas* infection include anyone with cystic fibrosis, tissue with burn wounds, or acute leukemia and who are in-patients in the hospital for an extended period of time (Sainz-Mejias et al. 2020).

The resistance mechanisms of *Pseudomonas aeruginosa* include the production of  $\beta$ -lactamases, efflux pumps, and target-site or outer membrane modifications (Zavascki et al. 2010). Thus, *Pseudomonas* has been listed as an infectious pathogen, and alternative therapeutics against this infection and its drug resistance are the absolute necessity.

Antimicrobial peptides (AMPs) are components of the immune system that are either derived or synthesized from various organisms. Even in pathogens with high resistance, these peptides have maintained their potency over time (Yount et al. 2006; Velayutham et al. 2022). This indicates that the host-pathogen relationship has important coevolutionary influences. Cationic AMPs are currently being considered as possible antibiotic substitutes to help combat the issue of antimicrobial resistance. Despite having a wide range of lengths, amino acid compositions, and secondary structures, all peptides have a unique membrane-bound amphipathic conformation that can be attained. Recent research has shown that they accomplish their antimicrobial activity by interfering with a number of crucial

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cellular functions. Even multiple mechanisms can be used by some peptides (Nguyen et al. 2011). Typically, antimicrobial peptides showed broad spectrum of biological activity against gram-positive and gram negative bacterial species, despite their diverse origins, small size, amphipathicity and cationic characteristics (Raju et al. 2021). AMPs are positively charged proteins that range in size from 15 to nearly 50 amino acids. The amphiphilic nature of AMPs enables them to interact and disrupt the bacterial cell membrane. The established beta-lactams and glycopeptides antibiotics target penicillin binding proteins and the pentapeptide bridge to inhibit peptidoglycan synthesis and therefore cell wall synthesis (Makovitzki et al. 2006; Yount et al. 2006).

Our study focused on an AMP, RW20, which has been derived from histone acetyltransferases (HATs) of *Channa striatus* transcriptome library. This peptide has been synthesized, and its preliminary antibacterial activity has been screened using several bacteria. A recent report shows the antioxidant activity of RW20 in zebrafish larvae (Prabha et al. 2022). However, the role of RW20 to control the growth of *P. aeruginosa* is unknown and they were not tested in any clinical trial. Hence, this study evaluates the antibacterial and protective effect of RW20 using *in-vitro* and *in-vivo* (zebrafish larvae) models. The capability of RW20 to disrupt the bacterial membrane against the *P. aeruginosa* was investigated. Further, the mode of action of RW20 against *P. aeruginosa* was examined using field emission scanning electron microscopy (FESEM) and fluorescence assisted cell sorter (FACS) analysis. In the bacteria-infected zebrafish larvae, RW20 challenge led to a reduction in the bacterial infection-mediated stress and apoptosis. This study evaluates whether and how RW20 protects the infected zebrafish larvae from the bacterial infection and if the peptide can compromise the pathogen. Interestingly, as the zebrafish genome comprises of 70% of protein-coding human genes, and it has been widely used as a model for several human diseases, the outcome of this study has the potential to be considered for further clinical research (Guru et al. 2021).

## Materials and methods

### Disc diffusion assay

The preliminary antibacterial activity of RW20 was screened against a total of 6 different bacterial species such as *P. aeruginosa* ATCC 25668, *Bacillus cereus* ATCC 2106, *Escherichia coli* ATCC 9637, *Staphylococcus aureus* ATCC 33592, *Klebsiella pneumoniae* and *Micrococcus luteus* MTCC 6164. The efficacy of RW20 (10, 20, 30, 40 and 50  $\mu$ M) was evaluated against the above mentioned 6 bacterial strains at a load of  $1 \times 10^5$  CFU/

mL using the disc diffusion assay. After the treatment, incubated over night at room temperature. The zones of inhibition of RW20 against the bacterial species were estimated, and based on the effective zone of inhibition, the respective bacteria were chosen for further analyses. Based on the response of RW20 in the zone of inhibition (mm), the bacteria were grouped into completely susceptible (++++) (30 - 40 mm), highly susceptible (++++) (20 - 30 mm), moderately susceptible (++) (10 - 20 mm), less susceptible (+) (0 - 10 mm), and resistance (-) (0 mm).

### Antibacterial activity

Antibacterial activity of RW20 at different concentrations (10, 20, 30, 40 and 50  $\mu$ M) against *P. aeruginosa* was determined using minimal inhibitory concentration (MIC) assay. *P. aeruginosa* was cultured overnight and maintained in  $1 \times 10^5$  CFU/mL. The bacterial culture was then centrifuged to obtain a pellet, which was then exposed to RW20 in a 96-well plate. The bacteria and the peptide mix was incubated and maintained at 37 °C. After incubation, the absorbance of each well was measured at 600 nm (Raju et al. 2021).

### Protein leakage assay

To calculate the bacteria-induced protein leakage, *P. aeruginosa* culture was challenged with RW20, as described previously (Raji et al. 2019). RW20 at various concentrations (10 to 50  $\mu$ M) and 50  $\mu$ L of overnight grown *P. aeruginosa* ( $1 \times 10^5$  CFU/mL) were incubated in a test tube for 12 h. Following the incubation, samples were centrifuged for 15 min at 6000 rpm, and a 200  $\mu$ L supernatant was mixed with 800  $\mu$ L Bradford reagent. The untreated *P. aeruginosa* were used as a negative control and gentamicin treated to *P. aeruginosa* was used as positive control. The absorbance was determined at 595 nm and the protein content was quantified in the supernatant.

### Release of intracellular components

The intracellular components released by the bacteria due to the peptide challenge was identified as described previously (Chen et al. 2013). Release of the components from the cytoplasm was calculated as per the absorbance obtained at 260 nm. Briefly, in a 96-well plate, bacterial suspension ( $1 \times 10^5$  CFU/mL) was added and treated with various peptide concentrations (10 to 50  $\mu$ M). The untreated *P. aeruginosa* were used as a control. After 2 h of treatment, the sample was centrifuged for 5 min at 5000 rpm and the supernatant was isolated. Using an ELISA plate reader, the optical density of the supernatant was measured at 260 nm.

## Membrane destabilization assay

Using the 4',6-diamidino-2-phenylindole (DAPI) and Propidium Iodide (PI) dyes, the effect of RW20 treatment on *P. aeruginosa* membrane stabilization was investigated (Mendes et al. 2022). The bacterial culture, as  $1 \times 10^5$  CFU/mL, was treated with RW20 (50  $\mu$ M) for 20 min. Then the cells were washed with PBS and examined under a fluorescent microscope. The bacterial cells were then stained using PI (10  $\mu$ g/mL) followed by DAPI (20  $\mu$ g/mL). The DAPI stained every single cell nucleoid, whereas PI, a nucleic acid dye, stained the nuclear content of cells in which the cytoplasmic membranes are compromised. Untreated culture served as control, while the gentamicin (25  $\mu$ g/mL) exposed culture served as positive culture. The intensity of the PI stain was measured to calculate the dead cells using Image J software.

## Flow cytometry analysis

Flow cytometry analysis established the number of viable bacteria, with or without the peptide challenge. In brief, RW20 (50  $\mu$ M) treated *P. aeruginosa* culture ( $1 \times 10^5$  CFU/mL) was incubated for 12 h. Following the incubation, culture media was removed by centrifugation for 5 min at 5000 rpm. The dying cells or cells undergoing apoptotic condition can be identified in the flow cytometry based on the side light scatter (SSc) and the PI fluorescence was detected using the FL3 channel of flow cytometry (Frossard et al. 2016). The results were analyzed by comparing the shifts in the bacterial cells between R1 and R2.

## FESEM analysis

Changes in the morphology of *P. aeruginosa*, in the presence or absence of RW20 were observed using FESEM analysis (Raju et al. 2021). The mid-log phase bacterial cultures were incubated for 2 h at 37 °C with the peptide (50  $\mu$ M). Bacteria ( $1 \times 10^5$  CFU/mL) were then pelleted by centrifugation and fixed in 2.5% glutaraldehyde for 1 h. Further, the bacteria was air-dried and washed with different gradients of ethanol. Finally, the bacterial cells were visualized under FE1 quanta FEG 200 High-resolution SEM.

## Exposure of zebrafish larvae to *P. aeruginosa*

Zebrafish embryos were purchased from a commercial supplier (NSK aquarium, Kulathur, Chennai, Tamil Nadu). Four-days post fertilized zebrafish (dpf) larvae were infected with *P. aeruginosa* through the bath immersion method (Boopathi et al. 2022; Haridevamuthu et al. 2022; Lite et al. 2022). The bacteria in culture medium, in stationary phase, were centrifuged for 5 min at 10,000 rpm to obtain the

bacterial pellet. At a final concentration of  $3 \times 10^8$  CFU/mL, bacteria from the pellet were used to infect the zebrafish larvae in E3 medium. Bacteria and larvae were co-cultured for 6 h in a 6-well plate to induce the bacteria-mediated stress in larvae ( $n = 50$ /group). Healthy uninfected zebrafish larvae served as control, while the larvae infected with *P. aeruginosa* was considered as infected group, and the infected larvae treated with RW20 was considered as test group.

## Protective effect of RW20 on the infected zebrafish larvae

The protective effect of RW20 (50  $\mu$ M) was evaluated using the 4 dpf larvae, which were infected with *P. aeruginosa*. The untreated larvae were used as control. Larvae ( $n = 50$ /group) were co-treated with RW20 and *P. aeruginosa* and incubated for 24 h. Following the incubation, mortality rate and the formation of deformities were estimated (Na et al. 2009).

## Macrophage staining

A neutral red stain was utilized to localize the macrophage accumulation in the zebrafish larvae (Dey and Kang 2020). Larvae were treated and incubated with *P. aeruginosa* and RW20 (50  $\mu$ M) for 1 hr. Further, to obtain the optimal staining of macrophages, the infected larvae and the peptide exposed larvae were incubated with neutral red (2.5  $\mu$ g/mL) for 2 h. Stained larvae were anesthetized and visualized under the microscope to observe the macrophage migration.

## Enzyme activity assays

Zebrafish larvae, after being infected with the bacteria and treated with RW20 (50  $\mu$ M), were anesthetized with tricaine. Then, the larvae were homogenized and centrifuged at 5000 rpm for 15 min (Issac et al. 2021a, b; Velayutham et al. 2021). The supernatant was used to determine the biological activities of the enzymes (including superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GPx)) and nitric oxide (NO).

SOD activity of the larvae group was determined by the pyrogallol auto-oxidation method (Marklund and Marklund 1974). The supernatant of the larvae was added to the reaction mixture of pyrogallol (2.64 mM) and 1 mM EDTA in Tris-HCl buffer. The absorbance was measured at 420 nm and the SOD activity was calculated. One SOD unit (U) is the amount of SOD that inhibits 50% auto-oxidation of pyrogallol.

To calculate the CAT activity in the supernatant of the larvae (Ellerby and Bredesen 2000), 0.05 M of phosphate buffer and 0.03 M of H<sub>2</sub>O<sub>2</sub> solution were added. The change in the absorbance was recorded for 1 min at 240 nm. One

unit of catalase activity is defined as the amount of enzyme that decomposes 1 mM of  $H_2O_2$  in 1 min

A reaction mixture containing 150  $\mu$ M of NADPH, 1 mM GSH, and 100 mM sodium azide in potassium phosphate buffer was added to the supernatant to estimate the GPx activity. The absorbance was read at 340 nm for 1 min (Adeyemi et al. 2015).

NO was determined using the Griess method (Stuehr and Nathan 1989). The supernatant was mixed with Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid] and the absorbance was measured at 540 nm.

### Quantification of ROS, apoptosis and lipid peroxidation, post-infection

The extent of bacteria-mediated excess oxidative stress, apoptosis and lipid peroxidation were evaluated using the fluorescent dyes such as 2',7'-dichlorodihydrofluorescein diacetate (DCFDA), acridine orange and diphenyl-1-pyr-enylphosphine (DPPP) (Guru et al. 2021, 2022; Gopinath et al. 2021). In brief, the larvae were infected with *P. aeruginosa* and co-treated with RW20 (50  $\mu$ M) for 1 h. Then, the larvae were stained individually for different dyes, with DCFDA (20  $\mu$ g/mL), acridine orange (7  $\mu$ g/mL) and DPPP (25  $\mu$ g/mL), and kept in dark for 30 min. Excess stain in the larvae was removed by ice-cold PBS. Fluorescent images was captured using a fluorescent microscope which is equipped with Cool SNAP-Pro colour digital camera (Olympus, Tokyo, Japan). The fluorescent intensity was quantified using Image J software (Version 1.49).

### Statistical analysis

All experiments were performed in triplicates; results are represented as an average of the triplicates. GraphPad Prism sets (Ver 5.03) were used to perform one-way ANOVA and Dunnett's multiple comparisons.

## Results

### Antibacterial activity of RW20

RW20 peptide with different concentration was tested for the antimicrobial activity in various bacterial strains. *P. aeruginosa* was found to be completely susceptible (++++) to RW20, among the six bacteria tested, followed by *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Micrococcus luteus* (Table 1). RW20 at a relatively higher concentration (50  $\mu$ M) was found to have better bactericidal activity.

**Table 1** List of bacterial strains used to screen the antibacterial ability of RW20 peptide

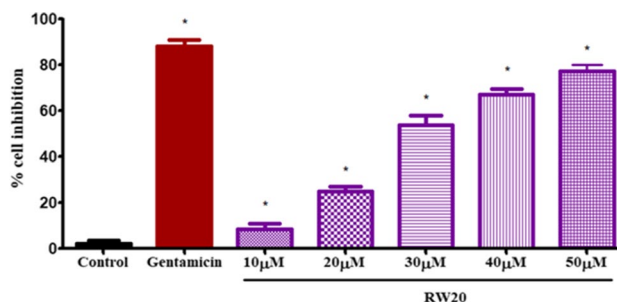
Bacterial isolates	Indication of sensitivity of RW20
<i>Bacillus cereus</i>	+
<i>Escherichia coli</i>	++
<i>Staphylococcus aureus</i>	+
<i>Klebsiella pneumoniae</i>	-
<i>Micrococcus luteus</i>	+
<i>Pseudomonas aeruginosa</i>	++++

### Effective inhibitory concentrations of RW20

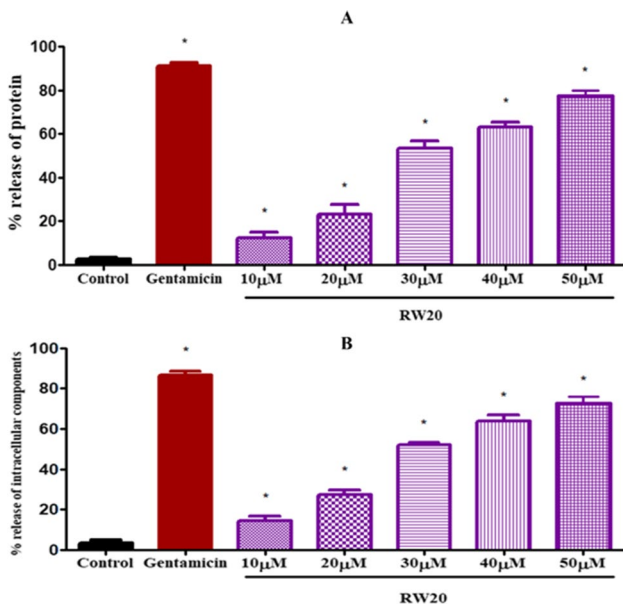
*P. aeruginosa* was completely susceptible to the RW20 treatment due to their effective antimicrobial activity. Further the effective inhibitory concentration on the different doses of RW20 was investigated. The MIC of RW20 against the *P. aeruginosa* was found to be at 30  $\mu$ M. While the maximum bactericidal concentration of RW20 was observed at 50  $\mu$ M against *P. aeruginosa*. The percentage of inhibition of various concentrations of RW20 against *P. aeruginosa* was shown in Fig. 1.

### Effect of RW20 on bacterial protein leakage and release of intracellular components

The amount of protein released during the RW20 treatment with *P. aeruginosa* was identified using the Bradford assay. The minimum amount of protein released from *P. aeruginosa* due to RW20 treatment was observed at 10  $\mu$ M (12.4%). While the maximum concentration, 50  $\mu$ M (77.2%) of RW20 showed a higher amount of protein release from *P. aeruginosa* (Fig. 2A). This observation suggests that RW20 is bactericidal, as it is toxic to *P. aeruginosa*, and that the peptide damages the bacterial cell membrane which leads to the release of cellular proteins.



**Fig. 1** MIC of RW20 at different concentration (10, 20, 30, 40, and 50) against PA where gentamicin was used as a positive control. Data were expressed as mean  $\pm$  SD. \* denotes  $p < 0.05$  as compared to the control



**Fig. 2** Release of microbial component on RW20 treatment at different concentration (10, 20, 30, 40, and 50 μM) against *P. aeruginosa*. The untreated *P. aeruginosa* were used as a control. **A** Optical density values were read at 595 nm for calculating release of protein. **B** Optical density values were read at 260 nm for calculating release of intracellular components. Values were shown as mean ± standard deviation of three replicates. The asterisk (\*) represents the significant difference at  $p < 0.05$

RW20 challenge-induced release of the intracellular components, from *P. aeruginosa*, was found to be elevated, in a dose-dependent manner. The untreated control has the least amount of release of intracellular components. While the

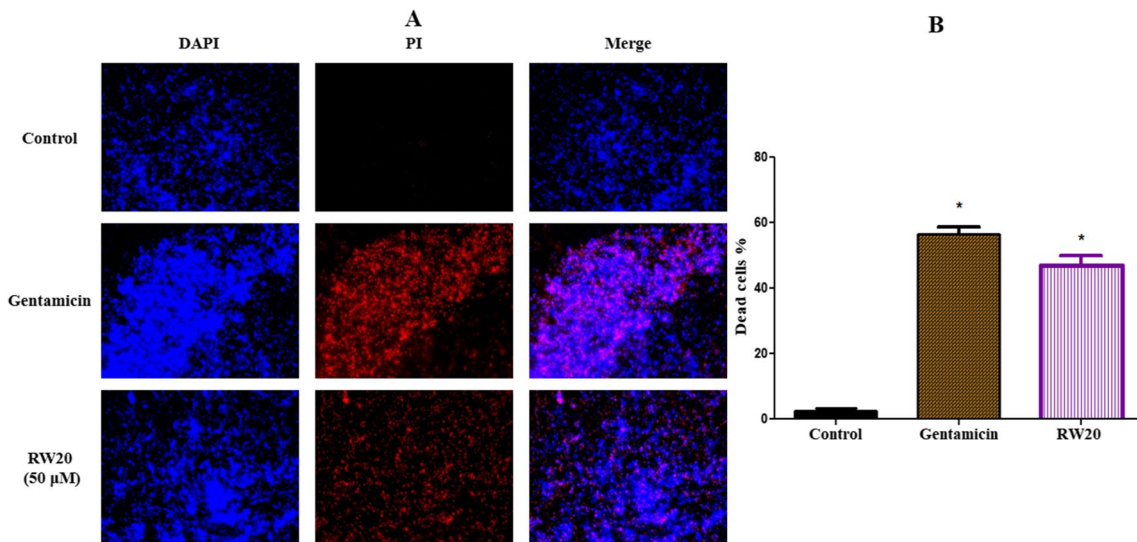
positive control, gentamicin, showed nearly 86% release of components from the bacteria. Besides, the maximum amount of DNA and RNA released in RW20 treatment was observed at 50 μM (72.5%) (Fig. 2B).

### Effect of RW20 on the bacterial cytoplasmic membrane

Disruption of the cytoplasmic membrane in *P. aeruginosa* after RW20 treatment has been investigated via the PI and DAPI dyes, combined staining approach, to visualize the live as well as dead cells. PI dye crosses the plasma membrane to bind with the DNA in the damaged cells. Results showed that untreated bacteria were stained blue (DAPI-positive), which indicates that the cells are alive (Fig. 3). Whereas, the bacterial cells treated with gentamicin or RW20, were stained red (PI-positive) due to damaged cell membrane. These images are merged to confirm the live and dead cells after the treatment. The percentage of death cells are measured using Image J software. The significant level of increase in cell death was observed in the bacterial culture after the RW20 treatment compared to the control group.

### FACS and FESEM analysis on RW20-mediated membrane permeabilization

The bacterial membrane permeabilization due to RW20 peptide was studied using FACS method. Based on the concentration of the RW20, the permeabilization in the bacterial cells was noticed by the intensity of the fluorescent shift from R1 to R2. FACS analysis, with FL3-height and SSc-height for 10s, showed a reduction (44%) in the number of *P.*



**Fig. 3** **A** Fluorescence microscopic images of *P. aeruginosa* stained with DAPI and PI after the treatment with RW20. The cells stained in blue (DAPI), while the cells with damaged membrane are stained

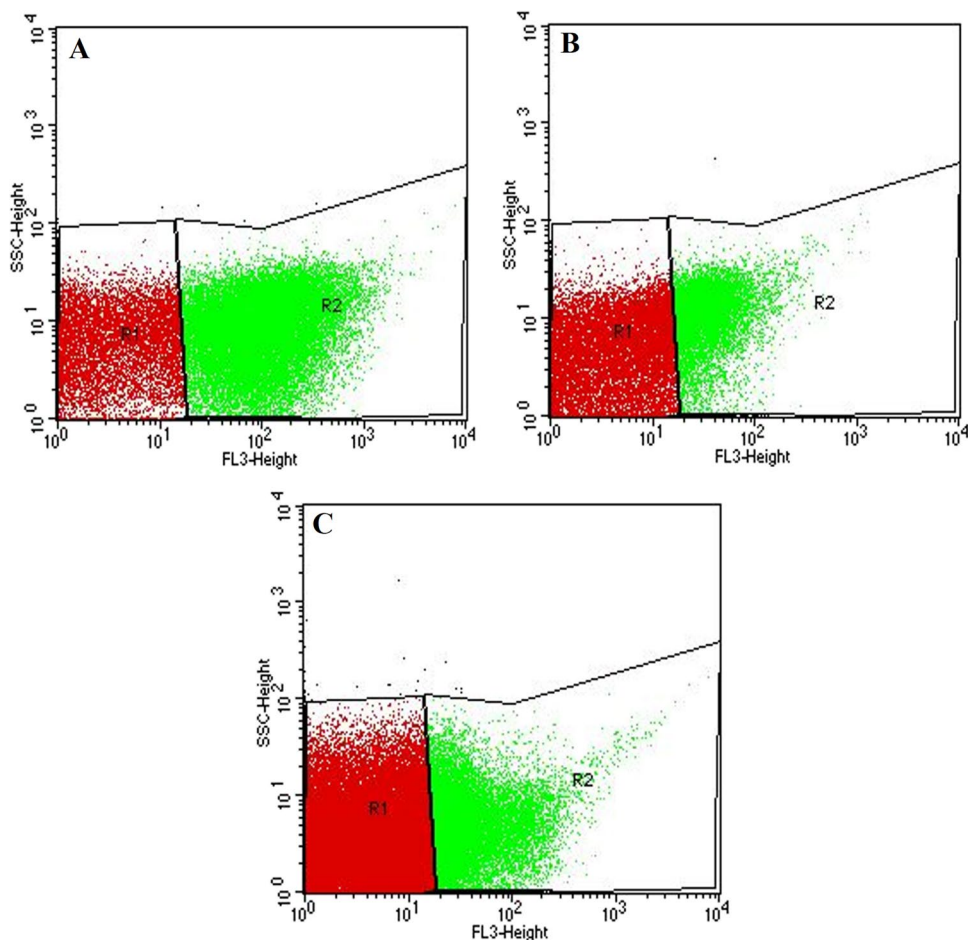
in red (PI). **B** The percentage of cell death after the RW20 treatment calculated in the PI stain. Using the Image J software, the fluorescent intensity of PI stain was calculated for cell death

*aeruginosa* due to RW20 treatment. Whereas the gentamicin challenge showed a significant decrease ( $p < 0.05$ ) in cell count (23%) when compared with the control (Fig. 4). The amphipathic nature of the peptide interact with the outer surface of the bacterial membrane which leads to membrane distraction. These changes in the bacterial cell membrane can be observed through the FESEM analysis. It was identified that RW20 challenge damaged the bacterial cell membrane while the untreated *P. aeruginosa* had normal smooth cellular surface without any membrane disruption (Fig. 5). These results suggest that RW20 peptide can kill the bacterial cells through damaging their cell membrane.

### Mortality rate and abnormalities in larvae

The protective effect of RW20 peptide was investigated in the infected zebrafish larvae. Mortality rate of the infected zebrafish larvae was reduced when the larvae were treated with RW20 (50  $\mu$ M) (Fig. 6A). Image analysis revealed that RW20 challenge, at 50  $\mu$ M, prevented the bacteria-induced morphological abnormalities, including bent spine and pericardial edema in the larvae (Fig. 6B and C). This result proves that RW20 can help to protect from the bacterial infection.

**Fig. 4** Quantitative cell count analysis using FACS analysis. **A** Untreated *P. aeruginosa* **B** *P. aeruginosa* treated with gentamicin (25  $\mu$ g/mL) and **C** *P. aeruginosa* treated with RW20 (50  $\mu$ M). The results are analyzed by comparing the viable cells (green colour) compared to the control



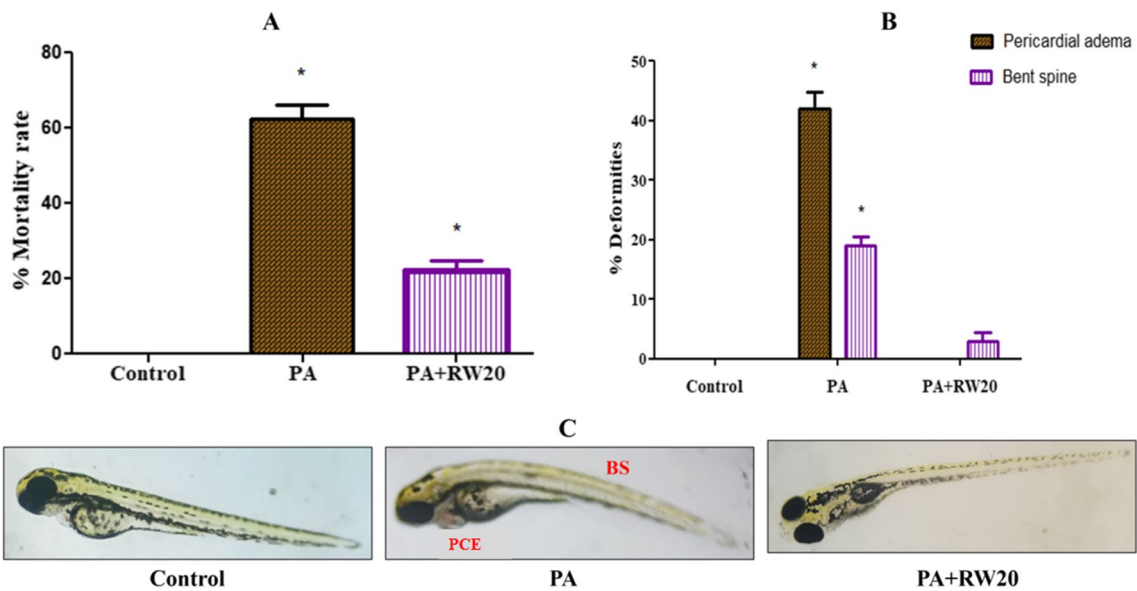
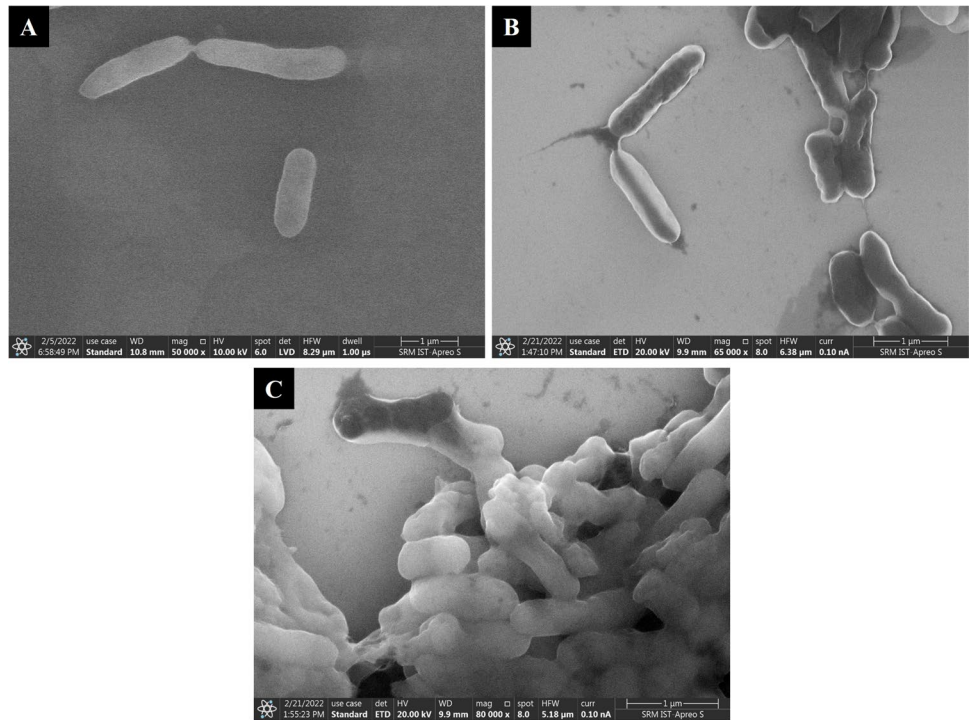
### Inhibition of macrophages migration

The bacterial infection in zebrafish larvae can lead to an inflammatory response. To investigate the inhibition of macrophage migration due to inflammation, a neutral red assay was performed. Macrophages were stained with neutral red and they appeared as dark-red aggregated granules. Results revealed that macrophage migration in zebrafish larvae was significantly higher in *P. aeruginosa* infected larvae than in the control (Fig. 7). Meanwhile, macrophages in the infected zebrafish larvae were less when treated with RW20 (50  $\mu$ M).

### Changes in the antioxidant enzymes and NO level in the infected zebrafish

During the pathogen infection, the host immune defense system of SOD and CAT enzymes is impaired. Similarly, the enzyme activities of SOD (11 U/mg of protein), CAT (2.5 U/mg of protein) and GPx (5 U/mg of protein) were reduced in the infected larvae compared to the healthy uninfected control (E-Suppl. Fig. 1A, B and C). However, the peptide challenge (at 50  $\mu$ M) increased the enzyme activities in the infected larvae, as SOD (20 U/mg of protein), CAT (5.6 U/mg of protein)

**Fig. 5** *P. aeruginosa* cells are observed for their morphological changes due to RW20 treatment under FESEM. **A** The FESEM image of untreated *P. aeruginosa* cells, which show smooth outer surface **B** Membrane disruption due to gentamicin (25 µg/mL) on *P. aeruginosa* cells **C** Membrane disruption due to RW20 on *P. aeruginosa* cells



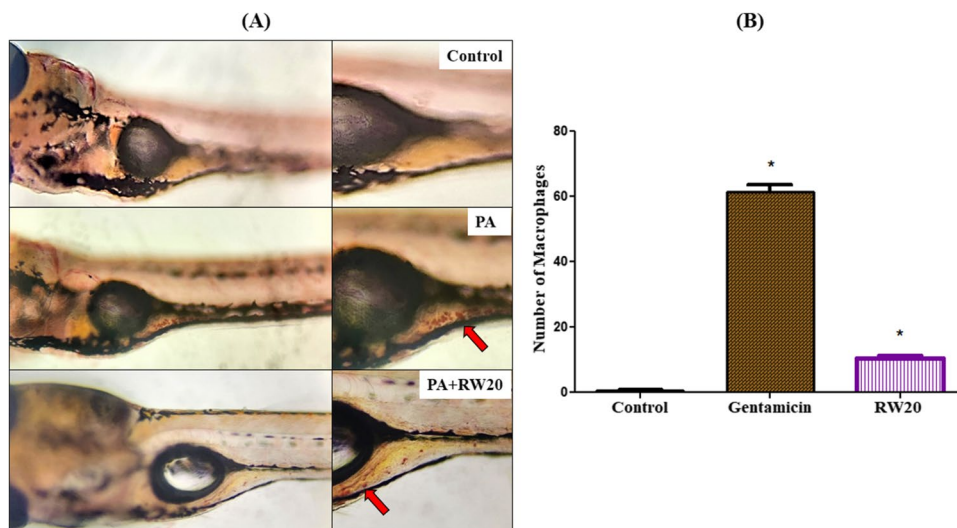
**Fig. 6** Effect of RW20 peptide on PA induced zebrafish larvae. **A** Mortality rate of larval zebrafish, **B** Percentage deformities of pericardial edema (YE) and bent spine (BS) larval zebrafish ( $n = 15$ ), and **(C)** Representative photograph of zebrafish larvae after treatment with RW20 peptide (50 µM). PA treated and untreated larvae are

used as positive control and control for the experiment, respectively. Experiments were performed in triplicate, and data were expressed as mean  $\pm$  standard deviation (SD) ( $n = 15$ /group). \* denotes  $p < 0.05$  as compared to the control

and GPx (12.5 U/mg of protein). This observation shows that RW20 reduces the bacteria-induced excess oxidative stress by enhancing the enzyme activities of the antioxidant system in the infected zebrafish larvae. Parallely, there was an increase

in the NO production in the infected larvae (46.5 µM), when compared with the control (E-Suppl. Fig. 1D). Meanwhile, the peptide challenge, at 50 µM, resulted a significant ( $p < 0.05$ ) restoration of NO level (27 µM).

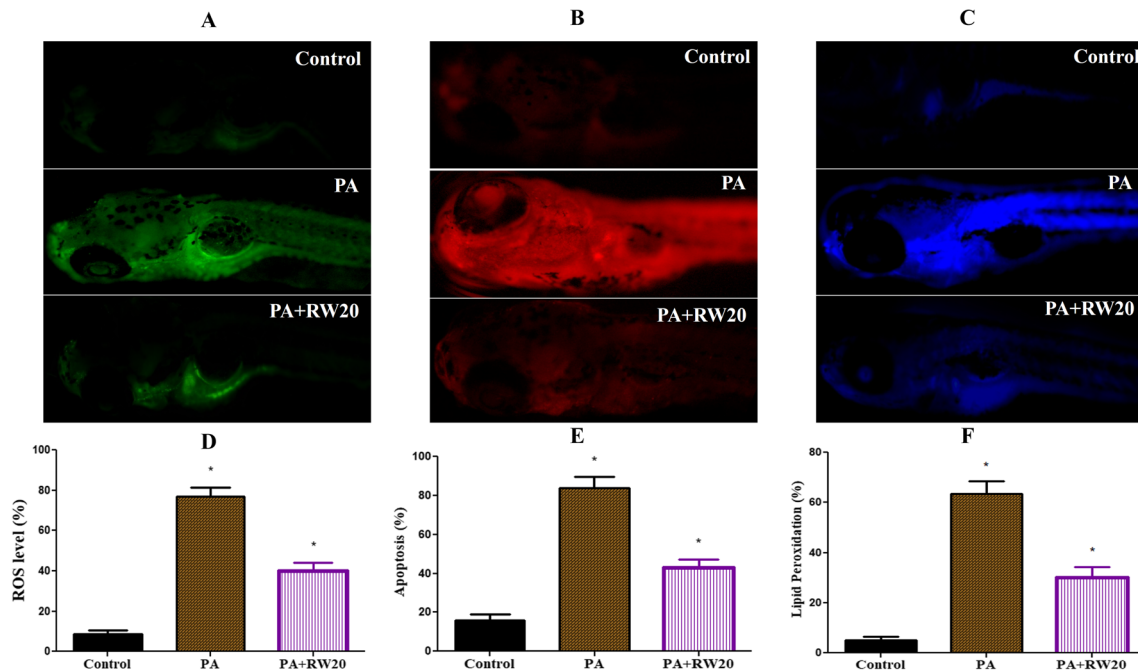
**Fig. 7** **A** Representative photomicrographs of zebrafish larvae stained with Neutral red staining. The accumulation of macrophages in the larvae was stained with neutral red. The red arrow marks the macrophages. PA, *P. aeruginosa*; PA+ RW20, *P. aeruginosa* + RW20 treatment. **B** The quantitative graph of number of macrophages in the zebrafish larvae



### Effect of RW20 against ROS, apoptosis and lipid peroxidation

The anti-inflammatory activity of RW20 against *P. aeruginosa* infected zebrafish larvae was investigated by DCFDA, acridine orange and DPPP. The infected larvae showed an increase in fluorescence intensity in DCFDA (77%), acridine orange (84%) and DPPP (63.5%), indicating an

increase in ROS, apoptosis and lipid peroxidation (Fig. 8). In the RW20 treated group (50  $\mu$ M), there was a decrease in ROS (40%), apoptosis (43%) and lipid peroxidation (30%), which implies that RW20 exhibited a protective anti-inflammatory role against *P. aeruginosa* that caused the inflammation in zebrafish larvae. These result suggest that RW20 can significantly normalize the microbial mediated inflammatory response.



**Fig. 8** Representative photomicrographs of zebrafish larvae due to **A** DCFDA **(B)** acridine orange and **C** DPPP staining. The fluorescence image was captured using a fluorescence microscope. Quantitative analysis of *in-vivo* **D** ROS level **E** apoptosis **F** lipid peroxidation

in zebrafish larvae. The fluorescence intensity was quantified using ImageJ. Experiments were performed in triplicate, and the data were expressed as mean  $\pm$  SD. The asterisk (\*) represents the statistical significance at  $p < 0.05$



## Discussion

*P. aeruginosa* is a Gram-negative aerobic bacterium that causes both community and hospital-acquired infections. Multidrug resistance has been observed in nosocomial *P. aeruginosa* infections. Earlier report states that *P. aeruginosa* can induce inflammation through oxidative stress and apoptosis (Boopathi et al. 2022). In our previous study, we have established the RW20 peptide, which has 20 amino acids and is rich in proline, valine, glycine, arginine, tryptophan and lysine. This peptide has been reported to have neuroprotective activity due to its effective antioxidant properties (Prabha et al. 2022). Typically, the antimicrobial activity has been highly influenced by the amino acid present in the peptide sequence. Reports suggest that presence of proline, arginine, glycine and lysine in the peptide sequence renders high antimicrobial activity against multidrug-resistant bacterial infection (Minami et al. 2004; Holfeld et al. 2018; Raju et al. 2021). Tryptophan containing AMPs have a unique ability to interact with the surface of bacterial cell membranes, potentially by improving antimicrobial properties (Bi et al. 2014). The bactericidal activity of RW20 was tested against a multiple of pathogenic bacteria, and *P. aeruginosa* showed lowest MIC of all organisms tested to the peptide. *P. aeruginosa* infection ranks third in the list of hospital-associated infections and is associated with chronic lung infections (Raju et al. 2021). Designing or developing an AMP for the prevention and treatment of *P. aeruginosa* involves complex processes. In this study, we have derived an AMP, from a natural source, teleost fish, that functions against *P. aeruginosa*. The RW20 against *P. aeruginosa* has been analyzed using minimal inhibitory concentration. Increasing concentrations of the peptide showed increasing inhibitory activity against the bacterial population. In support of this observation, a previous report (Bi et al. 2014) states that AMPs substituted with tryptophan amino acid kills the bacteria specifically by disrupting the cell membrane. The antibacterial activity of RW20 was also confirmed by the release of DNA, RNA and protein leakage from *P. aeruginosa* after treatment. The findings from the membrane integrity analyses were consistent with cell viability measurements, which implies that the reduction in bacterial survival rate could be associated with the damages to the bacterial membrane. An earlier study has reported (Wang et al. 2015) that ROS-induced oxidative damage leads to cell membrane damage, intracellular biopolymer leakage and cytoplasmic degeneration. In this study, a fluorescent PI assay differentiated the cells with compromised membranes from those with intact membranes (Dong et al. 2022). In our experiments, the fluorescent intensity of the bacterial cell was significantly enhanced after the treatment, indicating that the RW20 peptide destabilized the membrane. In FACS analysis, the viable bacterial cells were significantly ( $p < 0.05$ ) decreased after the RW20 treatment. To further observe the

structural and morphological changes in the bacteria, FESEM was performed. The untreated *P. aeruginosa* showed smooth cell surface, while the RW20 treated group showed damages on the surface of the bacterial membrane.

Zebrafish larvae have been used to study the pathogenesis of several infectious diseases. During the first week after fertilization, zebrafish larvae are transparent, allowing the researchers to clearly record the several infectious processes, using *in-vivo* imaging techniques (Saraceni et al. 2016; Sudhakaran et al. 2022). Zebrafish larvae were challenged with *P. aeruginosa* not just to understand the influence of the bacteria over morphological or cellular abnormalities and inflammation, but also to understand the protective effect of RW20. For zebrafish larval experiments, RW20 at 50  $\mu\text{M}$  was used since they are effective. The *in-vivo* zebrafish larval toxicity assay suggested that *P. aeruginosa* infected larvae showed reduced survival rate and had bacteria-induced abnormalities such as bent spine and pericardial edema in zebrafish larvae. This may be due to the onset of inflammation in the infected larvae. Co-treatment of RW20 in the *P. aeruginosa* infected larvae protects from developing abnormalities and significantly reduced the larval mortality rate. Reason may be the RW20 killed the bacteria, so the abnormalities would not form because of a lesser infection. Macrophages are one of the key components of innate cellular immunity, playing a key role in the host defense mechanism to protect against infectious bacteria. They have important secretory molecules that allow them to recognize, engulf and destroy the invading pathogens (Zhang and Wang 2014). Macrophage migration in the infected larvae was observed and compared with the untreated control. Results suggested that the macrophages are elevated in the infected larval group, which could be due to an increase in the inflammatory responses. Meanwhile, RW20 treatment reduced the macrophages, indicating a decrease in *P. aeruginosa* induced inflammatory response. Antioxidant enzymes are important in the host's immune defenses against the pathogenic microbes. SOD, CAT and GPx are some of the most common enzymatic antioxidants that scavenge the excess ROS, caused due to infectious bacteria (Shi et al. 2019). This study showed that *P. aeruginosa* infected larvae had reduced SOD, CAT and GPx antioxidant activities. However, the RW20 treatment group significantly ( $p < 0.05$ ) increased the enzymatic antioxidant level in the larvae to protect them from the *P. aeruginosa* mediated oxidative stress. Furthermore, excess NO, during inflammation, is a well-known pro-inflammatory mediator whose production is linked to immune stimulation. NO upregulation in this study strongly indicates the pathogen-induced cytotoxicity and host tissue damage. We also investigated whether the inflammation caused by *P. aeruginosa* is due to overproduction of NO (Sharma et al. 2007). Results indicate that NO production was elevated in *P. aeruginosa* infected group, while the RW20

reduced the NO production. ROS regulates the intracellular signal transduction events under physiological conditions, however, excess ROS causes oxidative stress in cells, resulting in apoptosis and lipid peroxidation (Sarkar et al. 2021). We have identified that bacterial infection triggered oxidative stress, apoptosis and lipid peroxidation, by using several fluorescent dyes such as DCFDA, acridine orange and DPPP. The fluorescence intensity was increased in the infected zebrafish larvae, indicating the augmentation of ROS, apoptosis and lipid peroxidation. But a significant ( $p < 0.05$ ) decrease in fluorescent intensity was observed in the RW20 treatment which shows that the larvae are protected from bacteria-induced oxidative stress. These findings suggest that the RW20 can act as an effective antibacterial agent that renders protection to zebrafish larvae from *P. aeruginosa* infection.

Our results indicate that RW20 exhibits both *in-vitro* and *in-vivo* antibacterial activity, providing an opportunity to explore its possible role against other bacterial pathogens. RW20 protected the larvae when they were co-treated with *P. aeruginosa*; however, further studies are required to elucidate the mechanisms which are involved in the anti-inflammatory activity of RW20 in reducing the oxidative stress and/or pro-inflammatory cytokines produced in response to the bacterial infection.

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**Author contributions** Conceptualization: AG and JA; animal experiment and data collection: AG and RM; formal analysis and original draft preparation: AG; review and editing: RM and JA. All authors have read and agreed to the published version of the manuscript.

**Data availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Code availability** Not applicable.

## Declarations

**Ethics approval** The fishes were collected, transported and handled for the experiment as per the Institute Animal Ethical Committee Guidelines and Approval (SAF/IAEC/211215/004).

**Informed consent** Not applicable.

**Consent for publication** Not applicable

**Conflict of interest** The authors declare that there is no conflict of interests.

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