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Antibacterial potential of different red seaweed (Rhodophyta) extracts against ornamental fish pathogen *Salmonella arizonae*

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ABSTRACT

This study evaluated the antibacterial effects of different red seaweed (*Kappaphycus striatus*, *Eucheuma denticulatum*, *Hydropuntia edulis*) against *Salmonella arizonae* that caused disease in goldfish *Carassius auratus*. *In vitro* antibacterial susceptibility was determined using a standard disc diffusion assay. Further *in vivo* experiments were conducted on seaweeds with the highest zone of inhibition. Results showed that *K. striatus* had the highest zone of inhibition with 30.9 ±0.62 mm followed by *H. edulis* (29.6 ±1.61 mm), and *E. denticulatum* (27.6 ±0.51 mm). Promisingly, the antibacterial activity of seaweeds tested was comparable with that of cefixime, trimethoprim, and novobiocin and was significantly higher than the other seven antibiotics tested in this study. Moreover, the *in vivo* treatment of *K. striatus* to *S. arizonae* challenged *C. auratus* significantly decreased the mortality; the positive control group attained 100% mortality while the treated group had 40% mortality after 10 days of post-infection. This study showed the potential use of *K. striatus* to control *S. arizonae* infection in aquarium fishes.

Keywords: Antibacterial, Bioassay, Seaweeds, Goldfish, *Salmonella arizonae*

Introduction

The emergence of multiple drug-resistant (MDR) pathogens has created a worldwide public health problem in the recent past. The concern on MDR has shifted the research priority of epidemiologists and allied researchers into the discovery of alternative sources of antimicrobial agents, wherein the zoonotic pathogen is of top concern. The development of MDR, moreover, is one of the major drivers of research to explore alternative natural antimicrobial agents that are locally available, cost-effective, and have minimal toxicity, but have lesser health impacts than commercial antibiotics (Cheung et al., 2014; Pérez et al., 2016; Cotas et al., 2020).

Seaweeds are marine plants without true leaves, stems, and roots and are mostly found on rocky shorelines (Cordero, 2009). Among the groups of seaweeds, red algae (Rhodophyta) have received much attention for it contains high amounts of polyunsaturated fatty acids, sterols, terpenes, mycosporine-like amino acids, essential amino acids, phycobiliproteins and carotenoids, and phenolic compounds (Torres et al., 2019; Cotas et al., 2020; Lopez-Santamarina et al., 2020). `In aquaculture, seaweed extracts have been used extensively for the prevention and treatment of viral and bacterial diseases (Noorjahan et al., 2022). Recently, the use of seaweed extract has received attention in pharmaceutical industries after research has revealed its inhibitory potential against the antibiotic-resistant pathogen (Cabral et al., 2021; Cotas et al., 2020; Klimjit et al., 2021; Lu et al., 2021)

Salmonella spp. is traditionally treated with antibiotics; however, recent reports on its antibiotic resistance have alarmingly increased (Wang et al., 2017; Cameron-Veas et al., 2018; Khademi et al., 2020). In the meta-analysis of Shen et al. (2022), it was revealed that *Salmonella* spp. isolates were highly resistant to tetracycline, sulfisoxazole, ampicillin, streptomycin, and sulfamethoxazole. This issue has contributed to the economic burden of most developing countries, where added costs are devoted to the prevention and treatment of persistent diseases caused by drug-resistance bacteria (Dodgostar, 2019). To date, research efforts and a vast literature has been published on the antibacterial activity of medicinal plant extracts against *Salmonella* spp., however, the focus was on common foodborne pathogens such as *S. typhi*, *S. enterica*, and *S. typhimurium* (Dayuti, 2017; Dhas et al., 2020; Martelli et al., 2020; Silva et al., 2020; Nozohour and Jalilzadeh, 2021; Gavriil et al., 2021; Wang et al., 2021; Naz et al., 2022). Meanwhile, studies on equally important health concerns and uncommon species and subspecies have remained elusive.

Salmonella enterica subsp. *arizonae* (Caldwell and Ryerson, 1939) is an uncommon pathogen initially described as a pathogen of cold-blooded animals, especially snakes until infection on humans, poultry, and fish have been published (Caldwell and Ryerson, 1939; Seligmann et al., 1944; Jortner and Larsen, 1984; Kodama et al., 1987; Hoag and Sessler, 2005; dos Santos et al., 2019; Limbago et al., 2021). By then, *S. arizonae* was considered zoonotic, mediating diseases in a wide array of animal species. In 2017, Nishioka et al. (2017) reported that *S. arizonae* developed resistance to the acceptable antibiotic, where there is recurring pyelonephritis secondary to *S. arizonae* infection even after cephalosporin treatments.

In 2018, a concerning record of *S. arizonae* infection was reported in *Carassius auratus* (Linnaeus, 1758) in the Philippines (Limbago et al., 2021). Mass mortality of *C. auratus* from a nearby fish pet shop in Barotac Nuevo, Iloilo, Philippines has been noticed, and *S. arizonae* is inferred to be one of the causes after performing Koch's postulate. Despite the study of Gut et al. (2022) on the antibacterial potential of traditional kefir against *S. arizonae*, the utilization of this dairy product in aquaculture might be expensive. Furthermore, the use of commercial antibiotics in aquaculture is currently discouraged since antibiotic residue may lead to the development of bacterial drug resistance (Santos and Ramos, 2018; Albarico and Pador, 2019). It is imperative, therefore, to screen locally available materials as a possible source of antimicrobials against *S. arizonae*, which motivated this study. In this study, the antibacterial activity of three red macroalgae species ethanol extracts was screened against a zoonotic pathogen, *S. arizonae*. This study aimed to contribute and answer the lack of research on uncommon *Salmonella* serotypes. Moreover, the results will be beneficial to aquaculturists and hobbyists should *S. arizonae* infection occur.

Material and Methods

Seaweed Sample Collection

Seaweed samples were collected from the coastal areas of Estancia, Northern Iloilo, Philippines. Collected samples were identified with the aid of the Field Guide and Atlas of the Seaweed Resources of the Philippines (Trono, 1997). Identified seaweed species were *Kappaphycus striatus* (F. Schmitz) Doty ex P. C. Silva (1996), *Hydropuntia edulis* (S.G. Gmelin) Gurgel and Fredericq, (2004), and *Eucheuma denticulatum* (N.L. Burman) Collins and Hervey, (1917). Seaweeds were then washed with distilled water to remove the adherent soils and salts. Cleaned samples were oven-dried at 60°C for 72 h and were cut into pieces using sterile scissors.

Ethanolic Extract Preparation

Samples were soaked into 500 mL 80% ethyl alcohol in a 1 L capacity Erlenmeyer flasks. The flasks were covered with carbon paper and stored in a dark cabinet at ambient temperature for 72 h. Each extract obtained was separately filtered using a Buchner funnel lined with Whatman No. 1 filter paper (Manilal et al., 2009; Lavanya and Veerapan, 2011; Salem et al., 2011). Then, a 100 mL filtered extract was concentrated to about 20 mL using a thermostatic waterbath (Lavanya and Veerappan, 2011). The remaining pure extracts were stored in a dark cabinet and were used in subsequent analysis.

Preparation of McFarland Standards

A 15×10^8 CFU/mL MacFarland standard was prepared by mixing 5 mL of 1.175% Barium Chloride dihydrate ($BaCl_2.2H_2O$) with 95 mL of 1% sulfuric acid (H_2SO_4). The mixture was then vortex for 30 secs. On the other hand, 30×10^8 CFU/mL MacFarland standard was prepared by mixing 10 mL 1.175% $BaCl_2.2H_2O$ with 90 mL of 1% H_2SO_4 (Montaño et al., 2022).

Bacterial Isolation and Culture

Pure cultures of *S. arizonae* were isolated from moribund *Carassius auratus* (Linnaeus, 1758) that were brought to the laboratory. Moribund *C. auratus* was brought to the laboratory and was clinically diagnosed with abdominal dropsy, loss of scales, rotten caudal tail, nonintact internal organs, and pale gills and flesh. Pure cultures of *S. arizonae* were maintained in nutrient broth and incubated at 25°C for 48 h. Every 3 days, working cultures were transferred to fresh nutrient broth media. Before subsequent experiments, a loopful *S. arizonae* culture was aseptically transferred to Shigella-Salmonella agar (SSA). Colonies on SSA plates were aseptically transferred into 10 mL tryptic soy broth (TSB) in replicates until bacterial suspension and turbidity reached 15×10^8 CFU/mL (used for *in vitro* test) and 30×10^8 CFU/mL (used for *in vivo* tests) following 0.5 MacFarland Nephelometer Standard (Ruangpan and Tendencia, 2004). Cultures incubated at 25°C for 5 h were used for subsequent study.

Preparation of Impregnated Disc

Sterilized 6 mm Whatman No. 1 filter paper discs were used in this study. In sterilization, discs were placed in a Petri dish and autoclaved at 121°C for 30 minutes under 15 psi pressure (Hossain et al., 2012). Sterilized discs were then oven-dried for 48 h. Subsequently, five compact discs were immersed in either 10 mL antibiotics or algal extract. Discs were dried for 24 h in an oven at 45°C.

In Vitro Antibacterial Test

In vitro antimicrobial activity of three seaweeds and ten commercial antibiotics (as positive controls) against *S. arizonae* were conducted using the standard disc diffusion method (Ruangpan and Tendencia, 2004). Briefly, the pure culture of *S. arizonae* was lawn on Muller Hinton agar (MHA) plates and then was dried for 10 minutes. Prepared impregnated discs were then placed on the MHA surface using sterile forceps. Samples were incubated for 72 h at 25°C, and plates were kept in an inverted position.

Antibiotics used as positive controls were (1) 10 g Gentamycin, (2) 5g Ciprofloxacin, (3) 30g Vancomycin, (4) 10g Streptomycin, (5) 30g Chloramphenicol, (6) G 10 units Penicillin, (7) 5g Cefixime, (8) 2.5g Trimethoprim, (9) 25g Amoxycillin, and (10) 30µg Novobiocin.

Bioassay Test

Twenty-five healthy *C. auratus* with an average weight of 15 g were brought to the laboratory and were acclimatized for five days before the experiment. Fish were subdivided into five treatments comprising five fish per 20 L capacity aquarium. Samples were exposed to different concentrations of seaweed extract to determine the maximum allowable concentration (MAC). The treatment I was exposed to 50 ppm; Treatment II (100 ppm); Treatment III (200 ppm); Treatment IV (300 ppm); and Treatment V (500 ppm). Concentrations in bioassay tests were based on Thanigaivel et al. (2015). Daily fish mortality was recorded to determine the toxicity of the extract. The MAC of seaweed extract to the fish was used for the antibactericidal test.

In Vivo Antibacterial Test

Fifteen healthy *C. auratus* were used in the conduct of this experiment. Fish samples were subdivided into three groups. Positive control and treated groups were intraperitoneally injected with 100 µL of *S. arizonae* (30×10^8 CFU/fish) while the negative control group was injected with 100 µL of distilled water. After 72 h of post-infection, groups were exposed to different treatments. The treatment group was exposed to the MAC of *K. striatus* extract while positive control and negative control were not exposed to the extract.

Antibacterial activity was determined by recording the daily fish mortality for 10 days. A second experiment was conducted to validate the results, employing the same methodologies.

Data Analysis

Inhibition zone and mortality rates were determined and statistically analyzed. Data were presented as percentages and means with standard deviation. Statistical differences were computed using One-way ANOVA with Tukey's HSD posthoc test using SPSS (IBM SPSS 22).

Results and Discussion

As shown in Figure 1, *K. striatus*, *H. edulis*, and *E. denticulatum* have a high zone of inhibition against *S. arizonae*. Among these three species, *K. striatus* had the highest antibacterial activity against *S. arizonae* with an average zone of inhibition at 30.9 ±0.62 mm indicating the sensitivity of the pathogen to the marine algae extract. A promising result was also noted in *H. edulis* with an average zone of inhibition of 29.6 ±1.61 mm and *E. denticulatum* with 27.6 ±0.51 mm, respectively.

Furthermore, the zone of inhibition of *K. striatus*, *H. edulis*, and *E. denticulatum* is not significantly different from that of cefixime, trimethoprim, and novobiocin. However, the zone of inhibition of three seaweed ethanolic extracts is statistically higher than that of gentamycin, ciprofloxacin, vancomycin, streptomycin, chloramphenicol, penicillin, and amoxicillin.

Bioassay Test

The bioassay test of *K. striatus* extract in *C. auratus* was carried out at different concentrations. Treatments and concentrations include Treatment I with 50 ppm concentration, Treatment II with 100 ppm, Treatment III with 200 ppm, Treatment IV with 300 ppm, and Treatment V with 500 ppm. The result of this experiment showed that the fish survived with all the treatments after 10 days of monitoring; indicating the non-toxic effect of the marine algal extracts (data not shown). The maximum allowable concentration (500 ppm) was used for the subsequent *in vivo* antibacterial test.

In vivo Antibacterial Test

The effect of *K. striatus* extracts on *S. arizonae*-infected *C. auratus* showed promising results (Figure 2). The positive control group has 20% mortality on the 3rd day after 72 h post-infection. The mortality of the positive control group has further increased to 100% mortality on day seven of post-infection. The treatment group, on the other hand, has a late onset of mortality as compared to the positive control group, starting with 20% mortality on day seven. The mortality in the treatment group has plateaued and was maintained at 40% until the 10th day of the experiment. While negative control group injected with distilled water has 0% mortality throughout the experiment.

Furthermore, treated *K. striatus* treated *C. auratus* showed normal flesh, intact scales, normal caudal fins, intact internal organs, and normal gills (Figure 3A) while the untreated control group showed pale gills, loss of scales, rotted caudal fin, internal organs not intact, and pale gills (Figure 3B).

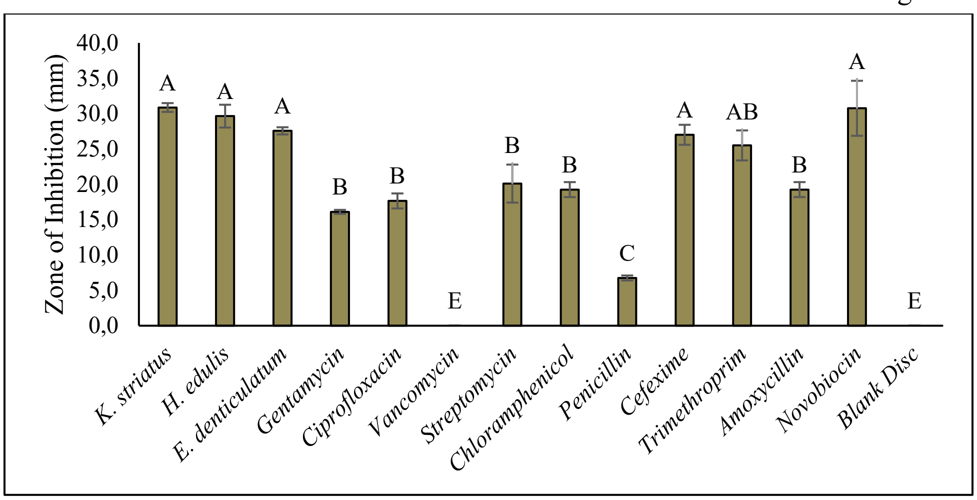

Figure 1. Susceptibility of *S. arizonae* to *K. striatus*, *H. edulis* and *E. denticulatum* ethanolic extract and thirteen commercial antibiotics. Each value is the mean zone of inhibition (mm) ± computed standard deviation from two replicates. Different superscripts mean the significant difference at $p<0.05$ levels.

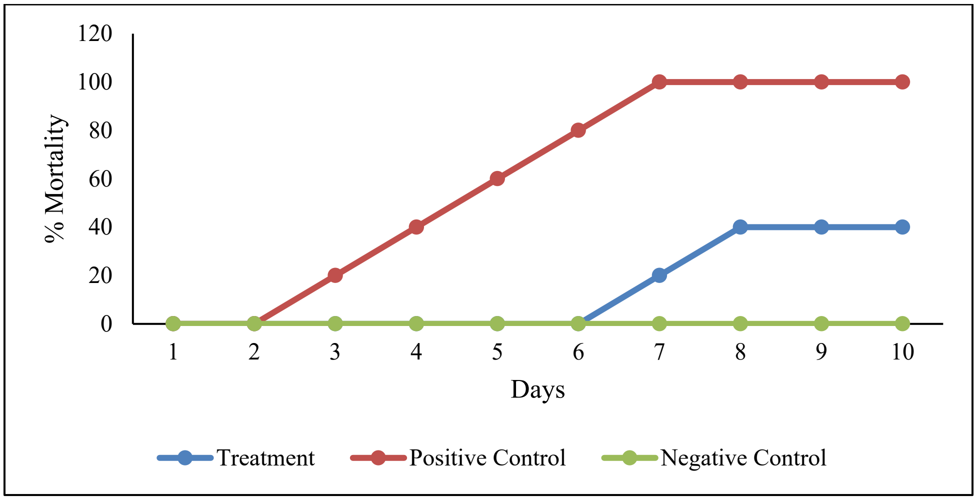

Figure 2. Antibacterial test of *K. striatus* ethanolic extract in *C. auratus* infected with *S. arizonae* for 10 days after 72 h post-infection.

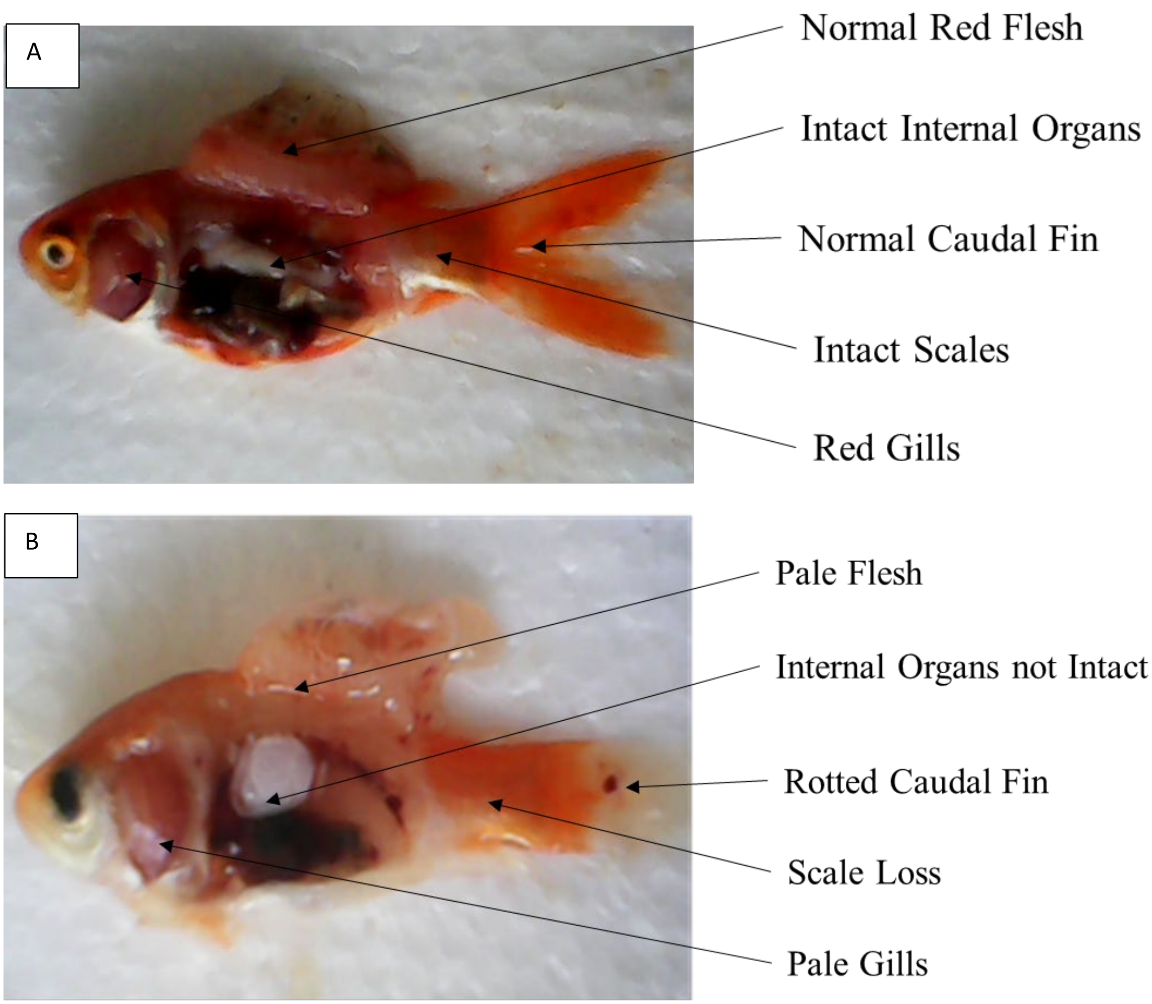

Figure 3. Dissected *C. auratus* infected with *S. arizonae.* (A) Treated group (B) Untreated control group.

In some Eastern countries, like the Philippines, marine autotrophs have been already used for their medicinal purpose. The diversity of plants and their traditional medicinal use has led to vast research to prove their therapeutic activity. Thus, it is worthwhile to conduct studies on life-saving drugs and biologically active substances from this renewable resource.

In the present study, *in vitro* experiments revealed that *K. striatus* has the highest inhibitory activity against *S. arizonae*. Promising antibacterial activity against *S. arizonae* was also observed from *H. edulis* and *E. denticulatum* extracts. This study also revealed that *K. striatus*, *H. edulis*, and *E. denticulatum* antimicrobial activities are comparable with tested commercial antibiotics. Although no studies have been conducted on the antibacterial effect of *K. striatus* on *S. arizonae*, a similar study has been tested on *S. typhi* (Prasad et al., 2013). However, compared to the antimicrobial activity of *K. striatus* in *S. typhi*, *S. arizonae* is highly susceptible to *K. striatus* ethanolic extracts (Prasad et al., 2013). Consequently, the ethanolic extract of *H. edulis* in this study has also a higher zone of inhibition compared to the study of Mahendran et al. (2021) on *H. edulis* against *Salmonella* spp. with only 22 mm. Meanwhile, *E. denticulatum* ethanolic extract in this study has a higher zone of inhibition in contrast with the study of Magallanes et al. (2021) with only a 13.2 mm zone of inhibition against *S. typhi*.

Many factors contribute to the differences in antimicrobial activity of plant extract in a pathogen. Factors include plant species and the solvent-extraction method used in the study (Sameeh et al., 2016). For example, in the study of Chuah et al. (2017) methanol extract of *K. alvarezii* has zero zones of inhibition against *S. enterica* which contradicts the results of this study where the high inhibitory activity of *K. striatus* was observed against *S. arizonae*. In the study of Prasad et al. (2013), results showed that *K. striatus* was slightly more effective than *K. alvarezii*. Moreover, ethanolic *K. striatus* extract showed a higher zone of inhibition than methanol extracts. It could be inferred that methanol and ethanol extraction-method, yielded different amounts of bioactive compounds although it should be noted that authors used different seaweed species. As support, several studies have shown that different extraction method has yielded different amounts of bioactive compounds. For example, the study by Bhuyar et al. (2020) reported that higher polyphenols were detected in the ethanolic extract of *K. alvarezii* than in hot water extract. In the study of Rebecca et al. (2013), it was concluded that ethanolic extraction was the best solvent for maintaining the active compounds in almost species of seaweeds. Ethanolic extracts of *K. alverii* include levoglucosenone, pyridinemethanol, 1,2,5-thiadiazole-3-carboxamide, and 4-[(2-chloroethyl) amino]-N-(2-hydroxyethyl)] (Bhuyar et al., 2020). It should be noted that a higher yield of bioactive compounds such as phytochemicals would likely result in a higher zone of inhibition. Phytochemicals are synthesized in response to microbial infection (Kumar and Pandey, 2013; Mierziak et al., 2014). Phenolic compounds, which are abundant in red seaweeds, have the property to disrupt the cellular membranes of microbes leading to their antimicrobial mechanism (Kumar and Pandey, 2013; Djouossi et al., 2015; Mishra et al., 2017; Cabral et al., 2021). Hence, the abundance of phenolic compounds would likely inhibit the growth of microorganisms including food-related pathogens. However, the exact mechanism of action of phenols is not yet fully understood at the cellular and molecular level (Chibane et al., 2019).

Meanwhile, a bioassay test revealed that 500 ppm of *K. striatus* tested is not toxic to *C. auratus*. It could be attributed to the fact that *K. striatus* is edible, thus, posing no to little toxicity in animals. This result enhances the promising antimicrobial activity of *K. striatus* against *S. arizonae*. Moreover, lower mortality was recorded in treated fish during *in vivo* experiments compared with 100% mortality in the untreated group. Another interesting result is that the treatment of *K. striatus* in infected fish delayed the onset of mortality.

While there is a myriad of studies on the antimicrobial activities of different plant extracts against *Salmonella* spp., the focus has been on the foodborne pathogen, while studies on *S. arizonae* are rather elusive. Previously published studies on the antibacterial activity of plants focused on foodborne pathogens including *S. typhi*, *S. enterica*, and *S. typhimurium* (Dayuti, 2017; Dhas et al., 2020; Sliva et al., 2020; Nozohour and Jalilzadeh, 2021; Gavriil et al., 2021; Wang et al., 2021; Naz et al., 2022). Recent studies on the susceptibility of *S. arizonae* only include the study of Limbago et al. (2021) and Gut et al. (2022) in mangroves leaf extracts and traditional kefir, respectively. While this study contributes to the aforementioned data gap, further research on the isolation of phytochemicals of *K. striatus* and its antimicrobial activity in *S. arizonae* is recommended. It is recommended to maintain the water physio-chemical parameters in the aquarium, and regular water exchange to prevent the occurrence of diseases from opportunistic pathogens like *S. arizonae*.

Conclusion

In conclusion, the present results showed that the ethanolic extract of *K. striatus*, *E. denticulatum*, and *H. edulis* possess antibacterial activity against *S. arizonae*, *in vitro*. Further *in vivo* experiment indicates that *K. striatus* extract can reduce the mortality of *S. arizonae*-infected *C. auratus*. The results of this study project the utilization of red seaweeds in treating *S. arizonae* infection in aquaculture.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential, or perceived conflict of interests.

Ethics committee approval: The authors declare that all international, national and institutional guidelines for the care and use of laboratory animals have been followed and complied with for this study.

Funding disclosure: -

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Disclosure: -

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