Journal of Fisheries
Volume 9 Issue 2 August 2021
Peer Reviewed | Open Access | Online First

eISSN 2311-3111
pISSN 2311-729X

Original Article

Antibacterial effects of mangrove ethanolic leaf extract against zoonotic fish pathogen *Salmonella arizonae*

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Manuscript history
Received 6 July 2020 | Revised 2 August 2021 | Accepted 8 August 2021 | Published online 15 August 2021

Citation
Limbago JS, Sosas J, Gente AA, Maderse P, Rocamora MN, Gomez DK (2021) Antibacterial effects of mangrove ethanolic leaf extract against zoonotic fish pathogen *Salmonella arizonae*. Journal of Fisheries 9(2): 92205.

Abstract
The emergence of multiple drug-resistant pathogens, affecting aquaculture and public health, has put the spotlight on alternative medicine research. This study was conducted to evaluate the *In vitro* and *In vivo* antibacterial activity of mangrove ethanolic leaf extract (MLEE) against *Salmonella arizonae* isolated from *Carassius auratus*. *In vitro*, antimicrobial activity of 10 mangrove species and 13 commercial antibiotics were determined using the agar diffusion method. MLEE with the highest antimicrobial activity were subjected to qualitative phytochemical tests and bioassay experiments. *In vivo* antibacterial activity of MLEE was assessed using *C. auratus* intraperitoneally injected with *S. arizonae*. Results showed that *Sonneratia alba* has the highest antimicrobial activity against *S. arizonae* followed by *Avicennia marina*, *A. officinalis*, *Sonneratia ovata*, *Rhizophora mucronata*, *Excoecaria agallocha*, and *Bruguiera cylindrica*. However, bacterial isolate was resistant to *A. rhumpiana*, *Scyphiphora hydrophyllacea*, and *Laguncularia racemosa*. Interestingly, *S. alba* has comparable antimicrobial activity with amoxicillin, trimethoprim, novobiocin, and cefixime. The activity of *S. alba* could be attributed to the presence of flavonoids, saponin, sterols, tannin, and terpenoids. Moreover, *S. alba* has reduced and delayed the onset of goldfish mortality infected with *S. arizonae*. Based on these findings, the *S. alba* MLEE, is a potential antimicrobial resource against *S. arizonae*.

Keywords: Antibacterial; *In vitro*; *In vivo*; mangroves; phytochemicals; zone of inhibition

1 | INTRODUCTION

Salmonella enterica subspecies *arizonae* is uncommon *Salmonella* species belonging to the family of Enterobacteriaceae. Caldwell and Ryerson (1939) initially considered *S. arizonae* as a pathogen for cold-blooded animals, especially snakes. Until Seligmann *et al.* (1944) reported the first *S. arizonae* human infection, with gastroenteritis as primary symptoms. Moreover, Jortner and Larsen

(1984) reported the *S. arizonae* infection in domestic poultry while Kodama *et al.* (1987) reported infection in *Arapaima gigas* pirarucu. The recent report of the occurrence of *S. arizonae* in farmed fish was also reported in Brazil (dos Santos *et al.* 2019). Hence, *S. arizonae* was able to mediate human, reptilian, avian, and fish diseases, and was considered zoonotic. In fish, *S. arizonae* leads to mortality secondary to septicemia (Kodama *et al.* 1987). In humans, most *S. arizonae* infections have a good prognosis and fewer complications (Lee *et al.* 2016), still, severe cases have been documented in children below seven years of age and immune-compromised adults (Smilack and Goldberg 1975; Caravalho *et al.* 1990; Mahajan *et al.* 2003; Di Bella *et al.* 2011), individuals with underlying diseases such as human immunodeficiency virus (HIV) infection (Casner and Zuckerman 1990) and even malignancy (Cortes *et al.* 1992). The most recent report of *S. arizonae* infection was reported in Nigeria, Japan, and Taiwan (Lee *et al.* 2016; Nishioka *et al.* 2017; Nuhu *et al.* 2017).

In the Philippines, *S. arizonae* infection in humans and other domestic animals has not been reported. In March 2018, five moribund goldfish *Carassius auratus* were brought to the College's Fish Health Laboratory from a local ornamental pet shop in Barotac Nuevo, Iloilo, Philippines. Researchers immediately examined the fish and performed Koch's postulate to establish a pathogen-disease relationship. One of the possible and potential causative agents of morbidity identified in the laboratory was *S. arizonae*.

At present, there is no standard treatment for *S. arizonae* infection, although development of nano-bodies for its detection was already initiated (Nishioka *et al.* 2017; Ahmed *et al.* 2020). In humans, several cases have been reported that *S. arizonae* is successfully treated with cephalosporins (Nowinski and Albert 2000; Hoag and Sessler 2005; Kolker *et al.* 2012). However, a published case in Japan reported that treatment with cephalosporins might not be enough leading to recurrence of pyelonephritis (Nishioka *et al.* 2017). This could be alarming both in public health and ornamental fish industry since some *Salmonella* strains are zoonotic and have developed resistance to cephalosporins, clavulanic acid, cotrimoxazole, gentamicin, nalidixic acid, norfloxacin, and other common antibiotics (Wang *et al.* 2017; Cameron-Veas *et al.* 2018). More so, that ornamental fish trade industry is one of the most common pathways of spreading of pathogens that are carried along during live fish transport. On top of that, the intermittent use of antibiotics as metaphylaxis in aquaculture has led to the emergence of antimicrobial resistance which has spread across the world and is considered as a very serious concern by the World Health Organization (WHO) and European Union (EU) (Watts *et al.* 2017; Santos and Ramos 2018; Miranda *et al.* 2018). Hence, paving the way for massive research on discovering novel antimicrobial agents from a natural resource.

Mangroves are tree halophytes growing in the brackish and coastal areas (Kathiresan and Bingham 2001). Because of its long usage in folklore medicine, pharmaceutical chemists have acknowledged the presence of natural products in mangroves that has possible therapeutic effects (Nabeelah *et al.* 2019). Several studies have been published on the chemical compounds present in mangrove plants and their antimicrobial properties against human and animal pathogens (e.g. Chandrasekaran *et al.* 2009; Abeysinghe 2010; Ravikumar *et al.* 2011; Saad *et al.* 2012; Harizon *et al.* 2015; Audah *et al.* 2018; Behbahani *et al.* 2018; Eswaraiah *et al.* 2019; Musa *et al.* 2019). Other potential pharmacological uses of mangrove plant extracts are also reported, including antiparasitic (Lopez *et al.* 2018), antidiabetic (Okla *et al.* 2019; Sachithanandam *et al.* 2019), antioxidants (Okla *et al.* 2019), and anti-cancer (Lopez *et al.* 2018).

Given the urgency of the problem and as part of the growing efforts of the college to provide technical support to the fisheries industry, this study was conducted. This could be used as a reference for alternative prophylaxis for ornamental fish should *S. arizonae* infection occur. The objective of this study was to evaluate the antibacterial activities of mangrove ethanolic leaf extracts against *S. arizonae*, *In vitro*, and *In vivo*. Specifically, to determine the antimicrobial activity of ethanolic leaf extracts from 10 mangrove species and 13 commercial antibiotics against *S. arizonae* isolated from *C. auratus*. Further preliminary qualitative phytochemical analyses and *In vivo* experiments were conducted for mangrove ethanolic extract with the highest antimicrobial activity to determine its anti-bactericidal effect against *S. arizonae* using *C. auratus* infection model. This study, with our thorough knowledge, is the first time to evaluate the antibacterial activity of mangrove ethanolic leaf extracts against *S. arizonae* - a zoonotic and uncommon fish pathogen.

2 | METHODOLOGY

2.1 Preparation of mangrove leaf ethanolic extracts (MLEE)

Leaves of 10 mangrove species were collected from coastal Barangays of Guintas and Sitio Lamintao, Talisay, Barotac Nuevo, Iloilo, Philippines on 5 April 2018. Fresh leave samples were then brought to Fish Health Laboratory, College of Fisheries and Aquatic Sciences, Iloilo State College of Fisheries. Mangrove species were identified using the handbook of Primavera *et al.* (2004). Identified mangrove species include (1) *Avicennia marina*, (2) *A. officialis*, (3) *A. rhumpiana*, (4) *Sonneratia alba*, (5) *S. ovata*, (6) *Rhizophora mucronata*, (7) *Excoecaria agallocha*, (8) *Scyphiphora hydrophyllacea*, (9) *Lumnitzera racemosa* and, (10) *Bruguiera cylindrica*.

Leaves were then washed in distilled water to remove adherent soil and salt contaminants. Then leaves were air-dried for an hour, oven-dried at 70°C for 72 hours, and were powdered using bullet blender. The powdered leaves (100 g) were soaked in 300 ml of 80% ethyl alcohol and were stored in a dark room for 72 hours. The solution was filtered using the Buchner funnel lined with Whatman No. 2 filter paper. Extracts (100 ml) were water bath at 60°C until 20 ml concentrated extract was left, this is to remove 80% ethyl alcohol from the solution; thus, pure plant extract remains.

2.2 Presumptive bacterial identification

Standard procedures on the presumptive identification of bacterial isolates from fish samples were performed following the methods of Tonguthai *et al.* (1999) and Ruangpan and Tendencia (2004). Supernatant from viscera of moribund goldfish was streak on McConkey Agar and Salmonella-Shigella Agar (SSA) and incubated at 25°C for 48 hours. Series of presumptive analyses were then conducted such as gram staining, biochemical tests (indole, methyl-red, Voges-Proskauer, Simmons citrate, hydrogen sulfide, motility), and fermentation of sugars (glucose, sucrose, mannose, lactose, D-xylose, D-sorbitol, L-arabinose, Trehalose).

2.3 Test organism and culture

Pure cultures of *S. arizonae* isolated from moribund goldfish were maintained in nutrient broth and incubated at 25°C for 48 hours. Every three days, working cultures were transferred to fresh nutrient broth media to assure purity. Before subsequent experiments, a loopful *S. arizonae* culture was aseptically transferred to SSA. Colonies on SSA plates were aseptically transferred into 10 ml Tryptic Soy Broth (TSB) in replicate until bacterial suspension and turbidity reach 1.5×10^9 CFU ml^{-1} (used for *In vitro* tests) and 3×10^9 CFU ml^{-1} (used for *In vivo* tests) following 0.5 MacFarland Nephelometer Standard (Ruangpan and Tendencia 2004). Cultures incubated at 25°C for five hours were used for subsequent study.

2.4 Preparation of MacFarland nephelometer standards

A 1.5×10^9 CFU ml^{-1} MacFarland standard was prepared by mixing 5 ml of 1.175% barium chloride dihydrate ($BaCl_2.2H_2O$) with 95 ml 1% sulfuric acid (H_2SO_4) in a test tube with constant stirring (Chapin and Lauderdale 2007). On the other hand, 3×10^9 CFU ml^{-1} MacFarland standard was prepared by mixing 10 ml 1.175% $BaCl_2.2H_2O$ with 90 ml of 1% H_2SO_4.

2.5 Preparation of impregnated disc

Sterilized plates were prepared for the 10 different mangrove leaves extract in duplicate, following the disc immersion method (Ruangpan and Tendencia 2004). Sterilized 6 mm diameter filter paper discs in five compact layers were impregnated with 10 ml MLEE by immersion technique in Petri plates. MLEE were oven-dried at 60°C until dried.

2.6 Susceptibility test

The standard disc diffusion method (Ruangpan and Tendencia 2004) was carried out to assess the susceptibility of *S. arizonae* on MLEE of 10 mangrove species and 13 common commercial antibiotics. Bacterial culture was lawn on Mueller Hinton agar (MHA) plates using a sterile cotton swab and were dried for 10 minutes. Then discs impregnated with different treatments: (1) MLEE for treatments, (2) antibiotics for positive control, and (3) blank disc for negative control. Discs were then placed on the MHA surface.

Antibiotics used as positive controls were (1) 2 g clindamycin, (2) 10 g gentamycin, (3) 5 g ciprofloxacin, (4) 10 units bacitracin, (5) 30 g vancomycin, (6) 10 g streptomycin, (7) 30 g chloramphenicol, (8) optochin, (9) 10 units penicillin G, (10) 5 g cefixime, (11) 2.5 g trimethoprim, (12) 25 g amoxycillin, and (13) 30 mcg novobiocin.

2.7 Preliminary phytochemical analyses

Species of mangrove with the highest zone of inhibition were subjected to preliminary qualitative phytochemical analyses. Methods of Khlif *et al.* (2015) were used to test the presence of alkaloids, flavonoids, saponin, steroids, tannin, and terpenoids.

2.8 *In vivo* experiments

Experimental animal: A total of 55 healthy, sub-adult goldfish, with an average weight of 25 g, were obtained from freshwater ornamental Biotech fish pet shop, Iloilo City, Philippines. Samples were divided into five fish per 20 L aquarium, provided with continuous aeration, and were acclimatized for five days before conducting the experiments.

Experiment I: sub-acute bioassay test: The MLEE with the highest zone of inhibition was used for this experiment. Healthy 25 sub-adult *C. auratus* were subdivided into five fish per 20 L aquarium. After acclimatization, fish samples were exposed to different concentrations of MLEE. Different treatment concentrations include: (1) 50 ppm, (2) 100 ppm, (3) 200 ppm, (4) 300 ppm, and (5) 500 ppm. Daily fish mortality was recorded to determine the maximum allowable concentration (MAC) of MLEE in *C. auratus*. MAC was used in the antibacterial test experiment.

Experiment II: antibacterial test: Fifteen goldfish were divided into three groups: (1) treated, (2) negative control and, (3) positive control group. Each group is consisting of five fish per aquarium in 20 L capacity aquarium. Treated and positive control groups were intraperitoneally (i.p) injected with 100 µl of *S. arizonae* (3×10^9 CFU $fish^{-1}$). The

negative control group was i.p injected with 100 µl distilled water. After 48-hour post-infection, goldfishes in the treated group were bath with 300 ppm *S. alba* ethanolic leaf extract. Daily observations of fish mortality were monitored for fifteen days to determine the antibacterial effect of the *S. alba* leaf extract infected goldfish. This experiment was conducted with two trials, employing the same methodologies.

2.9 Data analyses

Descriptive data analysis was performed. Unless specified, results were expressed as mean ± standard deviation (SD) from two trial experiments. All data analyses were performed and encoded with a statistical analysis system in MS Office Excel 365.

3 | RESULTS

3.1 Presumptive identification of bacterial isolate

Results of presumptive identification including morphology, gram staining, growth in selective media, and biochemical characteristics of *S. arizonae* are shown in Table 1. The isolate had a rod shape, a gram-negative, and growth was observed in SSA and McConkey Agar at 25°C. Positive results were observed in methyl red, Simmons citrate, H_2S production, motility, glucose (gas and acid), mannose, lactose, D-xylose, D-sorbitol, L-arabinose, Trehalose while the negative results were obtained from indole production, Voges–Proskauer and fermentation of sucrose.

TABLE 1 Biochemical characteristics of *Salmonella arizonae* isolated from goldfish *Carassius auratus*.

Parameters		Results
Morphology		Rod
Gram Staining		–
Growth in SSA		+
Growth in McConkey Agar		+
Indole Production		–
Methyl Red		+
Voges–Proskauer		–
Simmons Citrate		+
Hydrogen Sulfide production		+
Motility		+
Glucose, gas		+
Glucose, acid		+
Fermentation of	Sucrose	–
	Mannose	+
	Lactose	+
	D-xylose	+
	D-sorbitol	+
	L-arabinose	+
	Trehalose	+

+, positive results; –, negative results.

3.2 Susceptibility test

The result of the study revealed that MLEE showed promising antibacterial activity against *S. arizonae*, *In vitro* (Figure 1). Among 10 mangrove species tested, *S. alba* leaf extract had the highest antimicrobial activity against *S. arizonae* with an average zone of inhibition of about 23.5 ± 2.12 mm. This zone of inhibition was followed by *A. marina* (13.5 ± 0.71 mm), *A. officinalis* (13.5 ± 2.12 mm), *S. ovata* (15 ± 2.83 mm), *R. mucronata* (16 ± 2.41 mm), *E. agallocha* (12.5 ± 0.71 mm), and *B. cylindrica* (15 ± 1.41 mm) having an intermediate effect against the tested pathogen. However, *S. arizonae* was resistant to three of the other species, *A. rhumpiana*, *S. hydrophyllacea*, and *L. racemosa*.

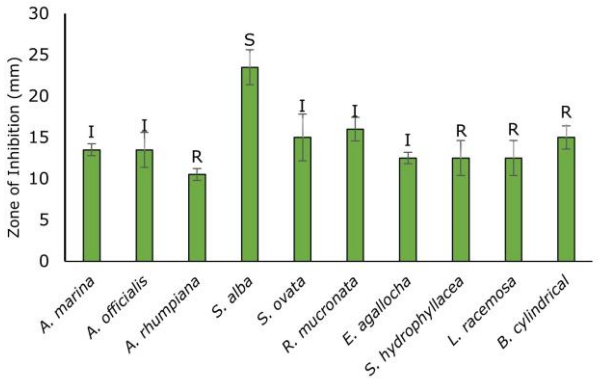

FIGURE 1 Susceptibility of *Salmonella arizonae* to 10 mangrove species ethanolic leaf extract. Each value is the mean zone of inhibition (mm) ± computed standard deviations from two replicates. R, resistant (≤13 mm); I, intermediate (14–18 mm); S, susceptible (≥19 mm).

The antibacterial of *S. alba*, moreover, was comparable to common commercial antibiotics. The *S. arizonae* was inhibited by cefixime, trimethoprim, amoxycillin, and novobiocin with a 20 – 28 mm range zone of inhibition (Figure 2). The test pathogen was moderately inhibited by clindamycin, gentamycin, ciprofloxacin, streptomycin, and chloramphenicol, while resistant to bacitracin, vancomycin, optochin and penicillin, *In vitro*.

3.3 Preliminary phytochemical analyses

The presence of phytochemicals and possible unknown bioactive compounds could be attributed to the antimicrobial effect of medicinal plants on pathogenic microorganisms. In this study, the ethanolic leaf extract of *S. alba* leaves have flavonoids, saponin, sterols, tannin, and terpenoids; however, free of alkaloids (Table 2).

3.4 Sub-acute bioassay toxicity

The ethanolic extract of *S. alba* exhibited toxicity on *C. auratus* at Treatment V, with an 80 % mortality (Table 3). While all other remaining Treatments, I, II, III, and IV, were non-lethal to fish with no mortality. Hence, 300 ppm, is the maximum allowable concentration (MAC) of *S. alba* in *C. auratus*, was used in the next *In vivo* antibacterial assay.

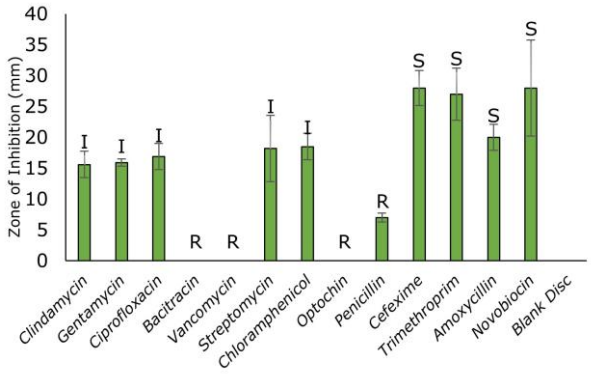

FIGURE 2 Susceptibility of *Salmonella arizonae* to common commercial antibiotics. Each value is the mean zone of inhibition (mm) ± computed standard deviations from two replicates. R, resistant (≤13 mm); I, intermediate (14–18 mm); S, susceptible (≥19 mm).

TABLE 2 Phytochemical analyses of *Sonneratia alba* ethanolic leaf extract.

Phytochemical	Tests	Results
Alkaloids	Mayer's reagent	–
Flavonoids	Shinoda, alkaline reagent	+
Saponin	Foam	+
Sterols	Chloroform and concentrated sulfuric acid	+
Tannin	10 ml of bromine	+
Terpenoids	Chloroform and concentrated sulfuric acid	+

+, positive results; –, negative results.

TABLE 3 Mortality records of goldfish exposed to different concentrations of *Sonneratia alba* ethanolic leaf extract.

Days of exposure	Treatments (ppm)				
	50	100	200	300	500
1	–	–	–	–	–
2	–	–	–	–	–
3	–	–	–	–	–
4	–	–	–	–	–
5	–	–	–	–	–
6	–	–	–	–	1
7	–	–	–	–	1
8	–	–	–	–	1
9	–	–	–	–	1
10	–	–	–	–	–
Total	0	0	0	0	4
Mortality (%)	0	0	0	0	80

3.5 Antibacterial bioassay test

The *In vivo* antibacterial activity of *S. alba* leaf extract was examined using the *C. auratus* infection model. *Carassius auratus* were infected with *S. arizonae* through intraperitoneal injection. After 48-hour post-infection, treated group was exposed to 300 ppm, the non-lethal dose of *S. alba* extract. As shown in Figure 3, mortality from positive control group started on day four with 20% mortality and has increased to 100% mortality in day fifteen. While treated group, has a late onset of mortality, beginning from 20% on the 8th day and maintains 40% mortality from 10th day until the 15th day of post-infection. The negative control group has zero mortality throughout the experimental period.

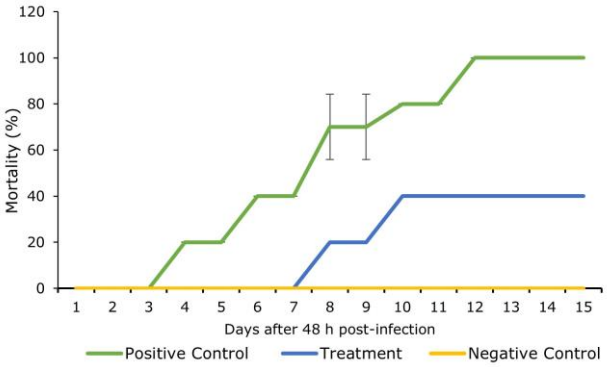

FIGURE 3 Percentage of mortality ± standard deviation from two trial experiments of *Carassius auratus* i.p injected with *Salmonella arizonae* (*n* = 5).

4 | DISCUSSION

Worldwide, the ornamental fish business is developing and considered one of the most lucrative in the aquaculture industry (Galib and Mohsin 2010; Walczack *et al.* 2017; Muyot *et al.* 2019). However, the unintended transport of pathogens along with the pet fish could lead to spread and may cause disease to a susceptible host (Smith *et al.* 2012). The spread and emergence of antimicrobial resistance (AMR) pathogen have put research of natural resource antimicrobial in the spotlight in the past decade.

Mangroves have been used as traditional medicine and claimed to be effective against human diseases (Thatoi *et al.* 2016; Nabeelah *et al.* 2019). Several studies have been established on the antimicrobial activity of different parts of mangroves against resistant human and animal pathogens to address the growing problem of the emergence of multiple drug resistance and zoonotic bacterial strains (e.g. Chandrasekaran *et al.* 2009; Abeysinghe 2010; Ravikumar *et al.* 2011). With this, it is significant to study the antibacterial effect of mangrove plants in the uncommon but drug-resistant pathogen, *S. arizonae*. Evaluating the antimicrobial properties of mangrove species, which is abundant in tropical regions is worthwhile, as it may lead to phytomedicine development against pathogenic microbes. In this study, the *In vitro* antibacterial activity of 10 mangrove species was tested against *S. arizonae* and was compared to 13 commercial antibiotics. Further *In vivo* experiments were conducted with *S. alba* ethanolic leaf extract using a piscine model infection.

The ethanolic leaf extract of *S. alba* had the highest zone of inhibition against *S. arizonae*, which is compara-

ble to *S. typhi* (Sahoo *et al.* 2012). Antibacterial properties of mangrove plants could be attributed to the presence of phytochemicals and some unknown bioactive compounds which might have a future clinical application (Bandaranayake 2002; Eswaraiah *et al.* 2019). In this study, preliminary phytochemical analyses detected the presence of flavonoids, saponin, sterols, tannin, and terpenoids in *S. alba*. Flavonoids, for example, are known antimicrobial agents against a broad spectrum of microorganisms (Xie *et al.* 2015). Interestingly, plants synthesized flavonoids in response to microbial infection (Kumar and Pandey 2013; Mierziak et al. 2014). The flavonoid mechanism of action, leading to its antimicrobial properties involves disruption of microbial membranes, nucleic acid biosynthesis inhibition, and microbial adhesins enzyme inactivation (Kumar and Pandey 2013; Djouossi *et al.* 2015; Mishra *et al.* 2017). However, it should be noted that ethanolic leaf extracts from different mangrove tested has varying antimicrobial effects against *S. arizonae*. There might be some unknown compounds that may explain its antimicrobial properties and diverse mechanism of mangrove species actions on *S. arizonae*, which is yet to be explained. For example, the study of Harizon *et al*. (2015) and Musa *et al.* (2019) identified new lupane-type triterpenoids from *S. alba* bark and leaf extract and has antibacterial activities against human pathogens.

The maximum allowable concentration of *S. alba* on goldfish was 300 ppm, this concentration was used for subsequent *In vivo* antibacterial assay. In the study of Avenido and Serrano Jr. (2012), the use of *S. caseolaris* as prophylaxis has no adverse effects on the histology of the *Penaeus monodon*. This present study and related literature suggest that *S. alba* is a promising source of phytomedicine and a favorable source of antimicrobials for aquaculture use. Moreover, the treatment of *S. alba* ethanolic leaf extract to *S. arizonae* infected goldfish, has reduced the mortality and delayed the onset of mortality, further increasing the promise of *S. alba* ethanolic extract as a treatment against *S. arizonae*.

This study, as the first study to test the efficacy of mangrove leaf extract against the uncommon ornamental fish pathogen, would be a great help to the hobbyist and eventually, the ornamental fish industry should *S. arizonae* infection would occur. Furthermore, the use of organic and natural resources may reduce the threat of multiple drug resistance. However, it should be noted that this study has limitations. First, only presumptive identification of *S. arizonae* was conducted, second, preliminary phytochemical analyses were performed, and lastly, the toxicity of *S. alba* in goldfish was conducted using a small sample size and inadequate replications. Therefore we recommend further PCR techniques be conducted to verify the isolation of *S. arizonae* and robust toxicity assay with adequate sampling size. With the promising antibacterial activity of *S. alba*, toxicity may also be evaluated on tissue and cellular levels.

5 | CONCLUSIONS

The present study revealed three primary results, (1) mangrove ethanolic leaf extracts, specifically *S. alba* extract, have antimicrobial activity against *S. arizonae* comparable to the commercial and standard antibiotics, (2) presence of flavonoids, saponin, sterols, tannin, and terpenoids were detected in *S. alba* ethanolic leaf extracts, and (3) *S. alba* ethanolic extract treatment in *S. arizonae* infected goldfish reduced and delayed the onset of mortality. Therefore, *S. alba* is a potential source of antibacterial phytomedicine against *S. arizonae*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

JSL & **JS** research design, field and laboratory experiments, manuscript preparation and revision; **AAG, PM, MNR** field and laboratory experiments; **DKG** research design and supervision, and review of the manuscript.

DATA AVAILABILITY STATEMENT

All data that support the findings of the study are available within the manuscript. Should any clarifications be needed, it could be requested from the corresponding author.

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