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In vitro Antimicrobial Activity of *Azadirachta indica* (Leaves) against Fish Pathogenic Bacteria Isolated from Naturally Infected *Dawkinsia filamentosa* (Blackspot barb)

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Abstract

To examine the antibacterial activity of ethyl acetate, ethanol and methanol extracts of *Azadirachta indica (A. indica)* on fish pathogens viz., *Aeromonas veronii, Aeromonas hydrophila, Acinetobacter junii, Acinetobacter tandoii, Acinetobacter spp.* and *Pseudomonas stutzeri* isolated from diseased blackspot barb, *Dawkinsia filamentosa*. The naturally infected fish, *D. filamentosa* were collected from Mettur Dam and the associated bacteria in the ulcerative lesions were isolated, sequenced the amplified product and tentatively identified through sequence similarity. The leaves powder was extracted successively with 250 ml of ethyl acetate, ethanol and methanol solvents by using Soxhlet extractor for 8 h at a temperature not exceeding the boiling point of the solvent. Ethyl acetate, ethanol and methanol extracts were subjected to phytochemical analysis as well as the antimicrobial assay using agar well diffusion method. Totally six fish bacterial pathogens were isolated from the ulcerative of lesions blackspot barb fish (*D. filamentosa*). All the gene sequences were deposited in the NCBI and received their accession numbers. The isolated strains were: A. veronii (KX688046), *P. stutzeri* (KX721473), *A. hydrophila* (KX756709), *A. spp*. (KX775221), *A. junii* (KX756708) and *A. tandoii* (KX775222). The different crude extracts of *A. indica* showed significant inhibitory effects on all the isolates. In which, the ethyl acetate extract showed maximum zone of inhibition on against *P. stutzeri* (23 mm) *A. junii* (22 mm), *A. hydrophila* (19 mm) and *A. tandoii* (18 mm). The ethanol extract showed moderate zone of inhibition on *A. junii* (15 mm) and *A. veronii* (14 mm) and also the methanol extract on A. junii (15 mm) and *A. veronii* (14 mm). The present study revealed that the crude extracts *A. indica* leaves possesses significant antibacterial activity on isolated fish pathogens. The susceptibility test was conducted on isolates using antibiotics, selected based on their importance to human medicine and on fish production. To conclude the present findings the ethyl acetate leaf extracts could be used as potential sources of antimicrobial agents.

Keywords: Fish pathogens; Antibacterial activity; Phytochemical analysis; *Azadirachta indica*; *Dawkinsia filamentosa*

Introduction

Aquaculture is a developing industry which needs sustained research with scientific and technical developments. In 2001, the world aquaculture production was approximately 37.9 million tons, which denotes around 41% of that gained from broad captures fishes for human consumption [1]. It requires adequate features in cultivating both freshwater and saltwater populations under controlled conditions, and can be contrasted with commercial fishing, especially harvesting of wild fish [2]. In aquaculture, the bacterial diseases cause severe economic losses [3]. The uncontrolled use of antibiotics to control pathogenic microorganisms causes undesirable changes in the microbiota of the aquaculture systems, surrounding environment and naturally beneficial bacteria were also disturbed [4,5]. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs [6]. The systematic intake of antibiotic treated fish by human can cause severe problems. Moreover, fishes used to produce immune compromised antagonistic to particular adaptable pathogenic bacteria [7,8]. The plant-based extracts are being treated against numerous fish diseases to induce innate behavior and immunity and also for controlling the spread of infection [9-14]. The plant-based extracts have been tried to control fish diseases, as a replacement for commercial antibiotics which defends the particular pathogenic bacteria against resistance [15,16].

In recent years a number of drugs, have been isolated from the medicinal plants according [17]. In developed countries, 80% of people use traditional medicine, that have been obtained from medicinal plants [18]. In both modern and traditional systems of medicines, the medicinal plants continue to serve as valuable therapeutic agents [19]. The *Azadirachta indica* (A. Juss.), belonging to the family, Meliaceae is a common large tree, is found throughout the Indian subcontinent. In Indian subcontinent, *A. indica* has been considered as the most abundant medicinal-plant for more than two millennia. In India, *A. indica* extensively used as a medicine in different fields like Ayurveda, unani and homoeopathic systems of medicine [20]. It leaves were widely used for control of leprosy, eye disorders, intestinal worms, fever, diabetes, and liver illness. The leaf extract showed superior antiviral and anti-hyperglycemic activity [21]. The plant-based extracts are nowadays used to treat and control the bacterial diseases rather than commercial antibiotics [15,16]. In Ayurveda, different parts of *A. indica* especially through preparations from leaves, fruits, flowers and barks were been used to cure several ailments [20]. Plant leaves possess activities like antimalarial, antibacterial, antifungal, antiviral, antipyretic, hypoglycemic, antiulcer, antifertility [20] as well as anticarcinogenic activity [22]. The anthelmintic properties of *A. indica* on domestic and economically important animals were earlier documented [23].

Cyprinid fishes of the species, *Dawkinsia filamentosa* are small in size, commonly called as black-spot barb and is widely distributed in Peninsular India [24]. It usually occurs in lower stretches of streams, rivers and river associated wetlands and swamps [25]. *D. filamentosa* is an important food and ornamental fish and is easily recognized by the presence of elongated, filament-like extensions of the branched dorsal fin-rays in adult males [26]. The present study, pertains to the antibacterial activity of different crude extracts of *A. indica* evaluated on isolated strains, from naturally infected *D. filamentosa*.

Materials and Methodology

Collection and maintenance of naturally infected fish

The diseased moribund freshwater fish, *D. filamentosa* (Figure 1) (N=10; SL= 75.8-106.5 mm) were collected from Cauvery Reservoir of Mettur Dam (11° 63'18" N, 77° 55' 15"), Tamil Nadu and were examined based on the clinical symptoms such as septicemia, hemorrhagic lesions, and ulcer on the skin surface of skin. The fish were transferred into laboratory condition within the 3 hours in an aerated polythene bag and maintained in 10 L aquarium tanks with tap water at a temperature of 30°C.

Figure 1: The naturally infected fish, *Dawkinsia filamentosa*.

Collection of medicinal plants

The healthy, disease free plant leaves of *Azadirachta indica* were collected from Mettur area, Salem district of Tamil nadu in May 2016.

Preparation of plant extracts

The freshly collected leaves were washed under the tap water and then distilled water to remove the external impurities. Then they were slice into small pieces and shade dried for two weeks and then blended into powder using mortar. The powdered leaves of *A. indica* was separately extracted with different solvents like ethyl acetate, ethanol and methanol by using Soxhlet apparatus for 24 h. The crude extracts were obtained by filtration using Whatman No. 1 filter paper and concentrated by using a rotary evaporator and used for further tests.

Phytochemical assays

The phytochemical analysis of ethyl acetate, ethanol and methanolic leaf crude extracts was carried to find the presence or absence of active different components or secondary metabolites such as tannins, alkaloids, flavonoids, and phenols by adopting standard protocols [27,28].

Isolation of bacterial strains from infected *D. filamentosa*

The ulcerative skin was dissected out from naturally infected fish and homogenized with sterile PBS (Phosphate Buffer Saline) buffer in a homogenizer. The sample was serially diluted to avoid overgrowth of bacteria [29]. Then, the diluted sample were inoculated onto different media likewise trypticase soy agar and nutrient agar by the spread plate technique [30]. The plates were incubated at 37°C for 24 hrs after the colonies were carefully examined and counted. Based on the morphological similarities, the colonies were selected and streaked in nutrient agar medium, until pure culture. Pure colonies were transferred into nutrient agar slant and stored at 4°C for further identification.

Molecular identification of bacterial strains by 16s rRNA sequencing

The bacterial strains were isolated from infected fish based on the morphological, biological, physiological and biochemical characters. Biochemical characterization of bacterial isolates was performed as described in Bergey's Manual of Determinative Bacteriology. The 16s rRNA sequencing of bacterial strains were subjected for further confirmation of pathogen. Genomic DNA was extracted and purified by using phenol: chloroform method.

Bacterial PCR and DNA sequencing

The 16s ribosomal RNA (16S rRNA) of isolated bacterial strains were amplified by polymerase chain reaction (PCR) with bacterial Universal primer. 30-cycle amplification was performed in a DNA thermal cycler (Eppendorf, Germany). For a 30 µL reaction: 15 µL of Taq DNA polymerase PCR master mix, 1 µL of amplified DNA, 1 µL of Universal bacterial forward primer (295267f)5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG 3', 1 µL of Universal bacterial reverse primer (295268r) -5'CACGGATCCTACGGGTACCTTGTTACGACTT 3' and 12 µL of milli-Q-water was added. DNA sequencing was done by direct sequencing of PCR amplified 16S rDNA gene after purification. DNA sequencing was performed in Eurofins Genomics, Bangalore, India.

Antibacterial activity testing

The antibacterial test was performed by agar well diffusion method of [31]. A sterile cotton swab was dipped into the 24 h old culture of bacterial isolates in nutrient broth inoculum, and then they were spread evenly on the nutrient agar plates. After the inoculum were allowed to dry under sterile condition for 10 mins. The 8 mm diameter wells were made in agar plate's distances of 3 cm on each well. The plates were kept under sterile condition for 10 mins to dry the inoculum. Into the well different concentrations (25,50,100,150,200 and 250 µl) of different crude extracts and in other well DMSO was added as a control. The plates were allowed to incubate for 24 h and the zone of inhibition was measured at the end of 24h.

Antimicrobial susceptibility tests

The antimicrobial susceptibility testing was done on Muller-Hinton agar by disc diffusion method (Kirby- Bauer diffusion susceptibility test). The following antibiotics with concentration ranges ciprofloxacin (5 µg), chloramphenicol (30 µg) and cephalexin (30 µg) were used. The six bacterial isolates separately tested against 3 antibiotics. The results were determined by using disk diffusion method as defined in the National Committee for Clinical Laboratory [32].

Results

Identification of pathogens

The screening of bacterial isolates was carried out based on their colony morphology, biochemical and molecular characterization. The biochemical characterization of the bacterial culture was performed (Table 1). Based on the biochemical characterization and morphology of the isolated bacteria from infected *D. filamentosa*, the isolates were tentatively identified as *Aeromonas veronii*, *Pseudomonas stutzeri*, *Aeromonas hydrophila*, *Acinetobacter spp.*, *Acinetobacter junii* and *Acinetobacter tandoii*. For further confirmation, the bacteria were analyzed by their 16S rRNA gene amplification using PCR. For this, bacterial universal primers 295267F and 295268R were used for the amplification of 16S rRNA gene and the results are shown in Figure 2. The nucleotide sequences were compared with the available sequence of species in the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) program. The sequences of the isolated strains discovered a homology of 99%. Hence, those were confirmed as *Aeromonas veronii* (KX688046), *Pseudomonas stutzeri* (KX721473), *Aeromonas hydrophila* (KX756709), *Acinetobacter spp.* (KX775221), *Acinetobacter junii* (KX756708) and *Acinetobacter tandoii* (KX775222) (Table 2).

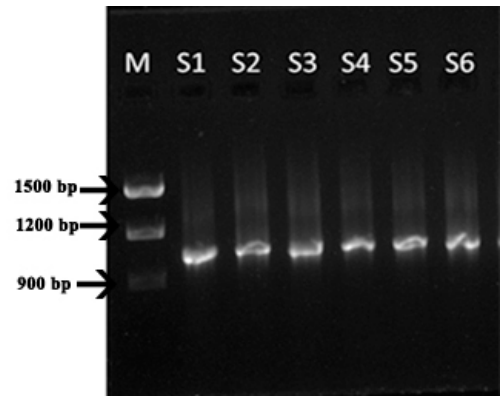

Figure 2: Amplification of DNA by PCR using bacterial universal primer. Lane 1: Marker, Lane 2: S1- *Aeromonas veronii*, Lane 3: S2- *Pseudomonas stutzeri*, Lane 4: S3- *Aeromonas hydrophilla*, Lane 5: S4- *Acinetobacter spp.*, Lane 6: S5- *Acinetobacter junii*, Lane 7: S6- *Acinetobacter tandoii*.

S.no	Biochemical tests	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6
1.	Shape	Rod	Rod	Rod	Bacilli	Bacilli	Bacilli
2.	Motility	+	+	+	-	-	-
3.	Gram staining	-	-	-	-	-	-
4.	Iodole	+	-	+	-	-	-
5.	MR	+	+	-	-	-	-
6.	VP	+	-	+	-	-	-
7.	Citrate utilization	+	+	+	-	-	+
8.	Catalase	+	+	+	+	+	+
9.	Oxidase	+	+	+	-	-	-
10.	Urease	-	-	-	+	+	-
Carbohydrate utilization							
11.	Glucose	+	+	+	+	-	-
12.	Lactose	-	-	+	-	-	+
13.	Nitrate reduction test	+	+	+	-	-	-

Table 1: Biochemical characteristics of the pathogenic strains isolated from infected fish. Strain 1: *A. veronii*, Strain 2: *P. stutzeri*, Strain 3: *A. hydrophila*, Strain 4: *Acinetobacter spp*, Strain 5: *A. junii* and Strain 5: *A. tandoii*.

Organ source	Isolates No.	Highest BLASTN match with bacteria	Similarity (%)	Genbank Accession number
Ulcerative lesions	1	*Aeromonas veronii*	99	KX688046
	2	*Pseudomonas stutzeri*	99	KX721473
	3	*Aeromonas hydrophila*	99	KX756709
	4	*Acinetobacter spp*	99	KX775221
	5	*Acinetobacter junii*	99	KX756708
	6	*Acinetobacter tandoii*	99	KX775222

Table 2: Identification of bacteria by 16S rDNA analysis, using PCR, and BLASTN data sequencing from Black spot barb (*Puntius filamentosa*).

Qualitative phytochemical analysis

The Phytochemical test revealed the presence of active chemical components such as Alkaloids, Phenols, Flavonoids, Saponins, Tannins, Carbohydrates, Protein, Quinones, Steroids and Glycosides. The results of qualitative phytochemical analysis of ethyl acetate, ethanol and methanolic leaf extract of Neem are given in Table 3. These phytochemical compounds have been described by [33] to inhibit bacterial growth and are capable of protecting certain plants against bacterial infections. The phytochemical analysis showed the Alkaloids, Phenols, Tannins and Steroids were found in all the crude extracts of *A. indica*, whereas the quinines is absent. The methanol extract of *A. indica* showed the presence of Flavonoids, Saponins, Tannins and Carbohydrates which are similar to ethanol extract.

Compounds	Methanol	Ethanol	Ethyl acetate
Alkaloids	++	+	++
Phenols	+	+	+
Flavonoids	+	+	-
Saponins	+	+	-
Tannins	+	+	+
Carbohydrates	+	+	-
Protein	-	-	+
Quinones	-	-	-
Steroids	+	+	+
Glycosides	+	-	-

Table 3: Phytochemical constituents of *A. indica* extracts. **Note:** Presence: (+), Absence: (-), highly present: (++)

Inhibitory effect of medicinal plants

The ethyl acetate, ethanol and methanol extracts of *A. indica* leaves were tested with five different concentrations (25, 50, 100, 150, 200, 250 µl) respectively, against the fish pathogens of *A. veronii*, *P. stutzeri*, *A. hydrophila*, *Acinetobacter spp.*, *A. junii* and *A. tandoii* respectively. In the present observation, ethyl acetate extract of *A. indica* showed maximum zone of inhibition on *Pseudomonas stutzeri* (23 mm) *Acinetobacter junii* (22 mm), *Aeromonas hydrophila* (19 mm) and *Acinetobacter tandoii* (18 mm). Whereas the ethanol extract resulted the moderate zone of inhibition on *Acinetobacter junii* (15 mm), *Aeromonas veronii* (14 mm) followed by the methanol extract, which also exhibited moderate inhibition zone on *Acinetobacter junii* (15 mm) Aeromonas veronii (14 mm), *Aeromonas hydrophila* (12 mm), *Acinetobacter tandoii* (12 mm), *Acinetobacter spp.*, (12 mm) and *Pseudomonas stutzeri* (12 mm). The antimicrobial assay results are described in Figure 3. Apart from these extracts ethyl acetate revealed the strongest antimicrobial activity in the all the strains when compared to other extracts at the concentration of 250 µl (100 mg/ml).

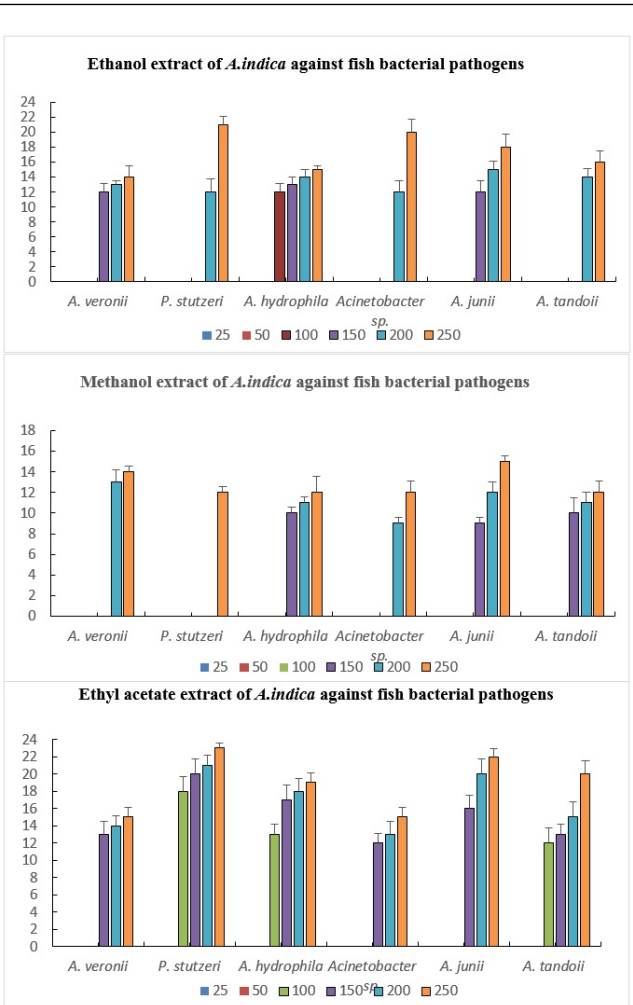

Figure 3: Antibacterial activity against fish associated pathogens through agar well diffusion method using different solvent extract of medicinal plant *A. indica*.

Inhibitory effect of standard antibiotic discs

The effect of different antibiotics provided useful information for the treatment of fish bacterial diseases under in-vitro condition. All isolated pathogens viz., *Aeromonas veronii*, *Aeromonas hydrophila*, *Acinetobacter spp.*, *Acinetobacter junii*, *Acinetobacter tandoii* and *Pseudomonas stutzeri* were significantly inhibited by Ciprofloxacin (24, 25, 11, 21, 31, 32 mm), Chloramphenicol (45, 13, 21, 22, 18, 20 mm), and Cephotaxime (44, 20, 12, 40, 28, 12 mm), respectively (Table 4).

Antibiotics	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6
Ciprofloxacin	24mm	25mm	11mm	21mm	31mm	32mm
Chloramphenicol	45mm	13mm	21mm	22mm	18mm	20mm
Cephotaxime	44mm	20mm	12mm	40mm	28mm	12mm

Table 4: Antibiotic susceptibility test performed against the 6 strains. Strain 1: *A. veronii*, Strain 2: *P. stutzeri*, Strain 3: *A. hydrophila*, Strain 4: *A. spp*, Strain 5: *A. junii* and Strain 5: *A. tandoii*.

Discussion

In India abundant work has been done on ethnomedicinal plants, as there has been an increased interest in a large number of traditional natural products of late. Several medicinal plants have been reported to possess antimicrobial, antifungal and other activity as explained by various workers [34,35]. Phytochemical extracts from Neem plant are potential sources of antiviral, antitumor and antimicrobial agents [20]. Several researchers have evaluated the antibacterial, antisecretory, antihemorrhagic, insecticidal activities of *A. indica* based drugs to meet the health care needs [36,37]. Almost every part of the tree was used in natural systems of medicine for the treatment of a variety of human ailments, particularly against diseases of bacterial and fungal origin [38].

In fish, the ulcerative lesions could form a portal of entry for opportunistic bacteria. Being able to detect and then culture from the earliest invisible lesions, would absolutely improve the ability to identify important pathogens, and therefore help in the diagnosis of bacterial diseases [39]. The importance of detecting early skin damage in bacterial infections has been demonstrated by some earlier researchers [40]. Also some previous researchers found the antibacterial activity against gram-positive and gram-negative bacteria with the methanolic extract of *A. indica* and P. guajava leaves [41,42]. And also, the methanol extract of neem leaf was tested for its antibacterial, antisecretory and antihemorrhagic activity against Vibrio cholera [37]. The aqueous extract of *A. indica* leaf has been tested against *A. hydrophila* infection in common carp, Cyprinus carpio and that showed this plant could effectively control *A. hydrophila* infection. In line with the earlier reports of several researchers, the results of the present study suggest that neem extracts possess compounds containing antibacterial properties that can potentially be useful to control fish pathogens [43]. The antibacterial activity of *Azadirachta indica* might be due to presence of triterpenoids, phenolic compounds, carotenoids, steroids, valavinoids, ketones and tetra-triterpenoids azadirachtin [36]. Compared with earlier authors report, the present study, revealed promising zone of inhibitions from leaves methanol extract on *A. junii* (15 mm) followed by *A. veronii* (14 mm), *A. hydrophila* (12 mm), *A. tandoii* (12 mm), *Acinetobacter spp.*, (12 mm) and *P. stutzeri* (12 mm), respectively.

Phytochemical extracts of Neem plant are being used as the sources of antitumor, antiviral and antimicrobial agents [20]. The results showed the presence of alkaloids, flavonoids, steroids, tannins, Phenols, Carbohydrates, Protein, Protein and Glycosides, as reported by some previous researchers [44-46], who have described the presence of phytocomponents in *A. indica* and cinnamon spice. These phytocomponents are considered as useful phytocomponents that not only enhance the action of anti-infla mmatory, antidiabetic, analgesic, central nervous system activities and nutritional value in the system but also act against disease causing organisms, thus conferring potential benefits by protecting from diseases.

Presently, the *A. indica* leaves ethyl acetate extract exhibited the largest inhibition zone size from 12 to 23 mm against six fish pathogens. Similarly, the leaf aqueous extract of *A. indica* has been

tested against *A. hydrophila* infection in common carp and the results showed that this plant could control efficiently *A. hydrophila* infection [43]. In our study, the ethyl acetate, ethanol and methanol extracts of *A. indica* leaves were found to have high antibacterial activity against fish pathogenic bacteria, viz., *A. veronii*, *P. stutzeri*, *A. hydrophila*, *Acinetobacter spp.*, *A. junii* and *A. tandoii*. Similarly, earlier the methanol extracts of Brazilian plants were found to exhibit the better antibacterial activity against fish pathogenic bacteria *S. agalactiae*, *F. columnare*, *A. hydrophila* and *F. columnare* [47]. The leaves *A. indica* extract exhibited high zone of inhibition against Enterobacter species and Escherichia coli isolated from marine fish, Amphiprion sebae that caused 15 mm zone [48]. The inhibitory activity of aqueous extract of pomegranate peel showed good activity against *P. stutzeri* [49]. Likewise, the hydroalcoholic extracts of Momordica charantia (100, 75, 50 and 25% methanol, hexane and water) were reportedly exhibited maximum activity against *Pseudomonas stutzeri* [50]. The ethanol extracts of musk lime, key lime and lemon exhibited significant inhibitory activity at 100% concentration (pure extract) when compared to water and juice extracts. 100% ethanol extracts of musk lime (39.7 mm), key lime (26.7 mm) and lemon (32.0 mm) exhibited high rate of inhibition against *Aeromonas veronii* when compared to earlier reports, the present analysis on ethanolic leaves extract of *A. indica* exhibited good zone of inhibition against fish pathogenic bacteria; *P. stutzeri* (21 mm), *Acinetobacter spp.*, (20 mm), *A. junii* (18 mm), *A. tandoii* (16 mm), *A. hydrophila* (15 mm) and *A. veronii* (14 mm), at 250 µl [51].

In the present study, six predominant bacterial strains were isolated from infected fish (*D. filamentosa*) through plating methods and they were identified as; *A. veronii, P.* stutzeri, *A. hydrophila*, *Acinetobacter spp., A. junii* and *A. tandoii*. The 3 different crude extracts of *A. indica* leaves at different concentrations were assessed for the inhibitory effects against the growth of fish bacterial pathogens and the ethyl acetate extracts of *A. indica* leaves were found to be more effective towards the bacterial species. The leaf ethanol extract was strongly active against bacterial pathogens except *A. veronii* and *A. hydrophila*. The leaf methanol extract of *A. indica* exhibited moderate inhibitory activity against the A. veronii, *A. hydrophila* and *A. tandoii* except *P. stutzeri* and *A. junii*. Among the different microorganisms tested maximum inhibition was found with ethyl acetate solvent extracts of *A. indica*. The leaves ethyl acetate extract can be used as alternative solution for the control of fish pathogenic bacterial strains.

Conflict of Interest Statement

We declare that we have no conflict of interest.

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