The Pharma Innovation Journal 2023; SP-12(10): 1155-1163

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(10): 1155-1163

www.thepharmajournal.com
Received: 22-08-2023
Accepted: 26-09-2023

Aabid Hussain Lone
Division of Fisheries Resource Management (FRM), Faculty of Fisheries, SKUAST-Kashmir, Rangil, Ganderbal, Jammu and Kashmir, India

Masood H Balkhi
Division of Fisheries Resource Management (FRM), Faculty of Fisheries, SKUAST-Kashmir, Rangil, Ganderbal, Jammu and Kashmir, India

Aadil Hussain Magloo
Division of Fisheries Resource Management (FRM), Faculty of Fisheries, SKUAST-Kashmir, Rangil, Ganderbal, Jammu and Kashmir, India

Rinkesh Nemichand Wanjari
Division of Fisheries Resource Management (FRM), Faculty of Fisheries, SKUAST-Kashmir, Rangil, Ganderbal, Jammu and Kashmir, India

Asim Iqbal Bazaz
Division of Fisheries Resource Management (FRM), Faculty of Fisheries, SKUAST-Kashmir, Rangil, Ganderbal, Jammu and Kashmir, India

Mudasir Ahmad Shah
Division of Fisheries Resource Management (FRM), Faculty of Fisheries, SKUAST-Kashmir, Rangil, Ganderbal, Jammu and Kashmir, India

Haziq Qayoom Lone
Division of Fisheries Resource Management (FRM), Faculty of Fisheries, SKUAST-Kashmir, Rangil, Ganderbal, Jammu and Kashmir, India

Corresponding Author:
Aadil Hussain Magloo
Division of Fisheries Resource Management (FRM), Faculty of Fisheries, SKUAST-Kashmir, Rangil, Ganderbal, Jammu and Kashmir, India

Antimicrobial activity of *Ceratophyllum demersum* against some fish pathogens in cold waters of Kashmir valley

Aabid Hussain Lone, Masood H Balkhi, Aadil Hussain Magloo, Rinkesh Nemichand Wanjari, Asim Iqbal Bazaz, Mudasir Ahmad Shah and Haziq Qayoom Lone

Abstract
The growing interest in plant-based medicines, considered safer and more reliable ("green medicine"), is steering a paradigm shift in combatting infections, especially in aquaculture. Medicinal plants, acknowledged for their antibacterial properties, present a compelling alternative to synthetic antimicrobials, potentially alleviating their detrimental effects. Aquatic plants, especially seaweeds, are emerging as robust reservoirs of bioactive compounds, sparking interest in allelopathic research. Seaweeds, teeming with diverse bioactive compounds, hold immense promise for the development of functional ingredients and therapeutic drugs. Their sophisticated chemical defense mechanisms, honed to combat microbial threats, augur well in the battle against human and fish bacterial pathogens, as well as various diseases. This study meticulously focuses on appraising the antibacterial potential of unique extracts derived from the submerged aquatic plant *Ceratophyllum demersum* (Coontail) against clinical isolates of fish bacterial pathogens. The research endeavors to offer invaluable insights into leveraging aquatic plants as a natural remedy to combat bacterial diseases in fish, emphasizing the promotion of sustainable practices within the aquaculture industry.

Keywords: Aquatic plants, aquaculture industry, bacterial diseases, *Ceratophyllum demersum*, seaweeds

Introduction
India ranks second globally in total fish production, boasting an annual fisheries yield of approximately 9.06 million metric tonnes. Within the European Union, farm animals consumed a significant 4,700 tonnes of antibiotics, constituting 35 percent of the total, while human consumption accounted for 8,500 tonnes, making up 65 percent. Among the antibiotics administered to animals, 3,900 tonnes (29 percent of the total) were used to aid ailing animals in their recovery, while 786 tonnes (6 percent of the total) were provided to farm animals to enhance growth. Notably, a survey estimated a 50 percent reduction in the use of antibiotics as growth promoters since 1997, when animals consumed approximately 1,600 tonnes as feed additives (FEDESA). In the domain of fish farming, encompassing aquaculture and mariculture, the widespread use of antibiotics to combat bacterial diseases has been linked to the emergence of antibiotic resistance in various aquatic pathogens, including *Aeromonas salmonicida*, *A. hydrophila*, *Edwardsiella icttaluri*, *E. tarda*, *Vibrio salmonicida*, *V. anguillarum*, and *Yersinia ruckeri* and *Pasteurella piscicida* [1]. Immediate and rigorous controlled studies are imperative to comprehensively evaluate the repercussions of antimicrobial therapy on the microorganism ecosystem within aquaculture ponds. The enthusiasm for plant-derived medications is growing, primarily due to the prevailing belief that "green medicine" is not only safer but also more reliable compared to synthetic drugs, many of which carry adverse side effects. The continual use of antimicrobial agents in aquaculture has led to the development of more resilient bacterial strains in aquatic environments. Prolonged reliance on synthetic antibiotics poses a looming threat to consumers and non-target organisms in the environment [2]. The treatment of bacterial ailments with diverse herbs has been safely employed in organic agriculture, veterinary medicine, and human healthcare [3]. Since ancient times, medicinal plants have played a pivotal role in treating common infectious diseases [4], and utilizing plants with antibacterial properties shows great promise as an alternative approach in aquaculture [2]. Medicinal plants offer effective means to combat infectious diseases while mitigating many of the adverse effects of synthetic antimicrobials [5].

Aeromonas hydrophila, the prevalent bacterial pathogen in freshwater fish, is known to be responsible for various pathological conditions, comprising tail/fin-rot and hemorrhagic septicemia, particularly in freshwater and ornamental fish [6]. Seaweeds inhabiting the depths of the ocean face a continuous threat from potentially dangerous co-existing microbes. To counter this, they have developed sophisticated chemical defense mechanisms by producing a diverse range of secondary metabolites [11]. These compounds, including fatty acids, alkaloids, glycosides, flavonoids, saponins, tannins, and steroids found in seaweeds, have exhibited notable effectiveness against various bacterial pathogens affecting humans and fish [12, 13], plant leaf spot diseases [14], and marine pathogenic microorganisms [15].

In India, a diverse range of medicinal plants has been traditionally harnessed to address a myriad of ailments. The exploration of bioactive substances within seaweeds presents a promising avenue for the development of novel therapeutic drugs. Extensive global research has focused on marine algae, resulting in the isolation of life-saving drugs and biologically active compounds [16, 17]. Reports highlight that more than 15,000 marine natural products were successfully isolated between 1965 and 2005 [18]. In this study, a variety of organic solvents have been employed to extract potential active compounds from aquatic plants. The primary objective is to uncover the antimicrobial activity exhibited by these selected aquatic plant extracts against bacteria and fungi. However, there remains a dearth of knowledge concerning the antimicrobial potential of hydrophytes from Kashmir, particularly as a natural remedy for fish bacterial pathogens. Thus, this investigation aims to evaluate the antibacterial potential of ethanol, acetone, methanol, petroleum ether, and water extracts obtained from the submersed aquatic plant *Ceratophyllum demersum* (Coontail), a member of the Ceratophyllaceae family. This research intends to shed light on the effectiveness of these extracts against specific clinical isolates of fish bacterial pathogens, paving the way for potential applications in aquaculture and related fields.

Materials and Methods

Plant material collection and identification

The aquatic plant species, *Ceratophyllum demersum* was collected from Dal Lake in Kashmir, India. An implicated specimen were retained in the form of herbarium of the division of Aquatic Environment Management (AEM) SKUAST-Kashmir, Faculty of Fisheries.

Preparation of extracts

A total of approximately 1044 grams of *Ceratophyllum demersum* was collected as a whole plant sample and underwent shade drying at a controlled temperature of 30±2 °C. After drying, the plant material was meticulously ground into a coarse powder using a grinder. Subsequently, five distinct extracts were prepared from *Ceratophyllum demersum* using different solvents, namely ethanol, petroleum ether, acetone, methanol, and water.

Petroleum ether extract

To prepare petroleum ether extracts, 50 grams of *Ceratophyllum demersum* powder were carefully placed into a thimble and subjected to extraction in a Soxhlet apparatus using 250 ml of petroleum ether. The temperature was precisely maintained within the range of 50-60 °C throughout the process. The contents within the thimble were allowed to boil for approximately 4 hours until the solution clarified, yielding a dark-colored extract that accumulated at the bottom of the apparatus. Subsequently, this extract was collected in petri dishes and left to air-dry for 24 hours, resulting in a dried extract with a sticky texture. The dried extract was then carefully transferred and stored in 25 ml McCartney bottles at 4 °C in the refrigerator for future use. This entire extraction procedure was continual three times to ensure an ample collection of extracts for the intended study.

Acetonic extract

To prepare acetonic extracts, 50 grams of Ceratophyllum demersum powder were meticulously packed into a thimble and underwent extraction in a Soxhlet apparatus using 250 ml of acetone. The temperature was carefully maintained within the range of 45-55 °C throughout the extraction process. The samples in the thimble of the Soxhlet apparatus were gently boiled for approximately 4 hours until the solution clarified, yielding a dark-colored extract that collected at the bottom of the apparatus. Subsequently, this extract was gathered in petri dishes and left to air-dry for 24 hours. The resulting dried extract, displaying a sticky texture, was then carefully transferred and stored in 25 ml McCartney bottles at 4 °C in the refrigerator for future use. The entire process was carried out three times to acquire a large number of extracts for the study.

Ethanoic extract

To prepare ethanoic extracts, 50 grams of *Ceratophyllum demersum* powder were carefully placed into a thimble and subjected to extraction in a Soxhlet apparatus using 250 ml of ethanol. The temperature was precisely maintained within the range of 60-80 °C. The samples in the Soxhlet apparatus thimble were gently boiled for nearly 4 hours until the solution turned clear, resulting in a dark-colored extract that accumulated at the bottom of the apparatus. Consequently, this extract was carefully collected in petri dishes and allowed to air-dry for 24 hours. The resulting dried extract, displaying a sticky texture, was then delicately transferred and stored in 25 ml McCartney bottles at 4 °C in the refrigerator for future use.

Methanolic extract

The preparation of methanolic extracts mirrored the process for ethanolic extracts, with the only variation being the use of methanol as the extraction solvent. 50 grams of powdered sample were packed into the Soxhlet apparatus with 250 ml of methanol. As the methanol boiled, the resulting extract was gradually collected at the bottom of the flask, maintaining a temperature range of 60-80 °C. Following overnight drying in petri dishes, the extracts were carefully gathered with a spatula and stored in McCartney bottles.

Aqueous extract

A quantity of 40 grams of *Ceratophyllum demersum* powder was accurately measured and combined with 280 ml of distilled water. The mixture was left undisturbed in a sterile environment for a period of 2 days. Subsequently, the liquid extract was filtered through Whatman Filter paper, specifically no. 40. The resulting filtrate was carefully placed in a water bath maintained at a temperature range of 80-90 °C until the extract underwent complete drying. Following this, the dried extracts were meticulously stored in McCartney bottles at a temperature of 4 °C.

Storage of extracts
The collection of extracts took place in 25 ml McCartney bottles, with each bottle being filled with the extracts. These extracts were dried until they exhibited a sticky, glutinous appearance. Various quantities of extracts were amassed. Once the extraction was completed and the extracts were gathered using a spatula, the McCartney bottles were securely stoppered and stored in the refrigerator at 4 °C.

Stock solution preparation for phytochemical assays
For conducting phytochemical assays, the extracts were dissolved to create a stock solution of 5 µg/L. This involved combining 0.5 grams of crude extract with 100 ml of solvent, resulting in a consistent method of preparing all five stock solutions.

Biochemical Assays
An initial screening of all five extracts was conducted using biochemical tests to identify various phytochemicals present in *Ceratophyllum demersum*. The crude extracts were examined for either the inclusion or absence of secondary metabolites such as alkaloids, phenolic compounds, steroidal compounds, saponins, tannins, flavonoids, and cardiac glycosides. To confirm the either the inclusion or absence of secondary metabolites in the plant extract, specific biochemical tests were performed. The detection of phytochemicals followed the methods outlined by Harborne [19].

Test for steroids
In a procedure, 0.5 ml of the solvent extract was combined with 2 ml of acetic acid, followed by the addition of 2 ml of H_2SO_4. The emergence of a blue or green color was considered indicative of the presence of steroidal compounds.

Test for tannins
For this step, 5 ml of the solvent extract was mixed with two drops of 5% $FeCl_3$. The appearance of a greenish precipitate served as confirmation for the presence of tannins.

Test for terpenoids
In this step, 5 mL of the solvent extract was mixed with 2 mL of chloroform, followed by 3 mL of concentrated sulfuric acid (H_2SO_4). The occurrence of reddish-brown colouring near the contact was interpreted as confirmation of the existence of terpenoids.

Test for flavonoids
A few drops of a solution containing lead acetate were incorporated into 2 ml of solvent extract. The presence of flavonoids was confirmed by the production of a yellow-colored precipitate.

Test for alkaloids
A small amount of picric acid solution was introduced into 2 ml of the solvent extract. The emergence of an orange color was taken as an indication of the presence of alkaloids.

Test for saponins
1 mL of solvent extract was placed in a tube with 1 mL of distilled water. After that, the liquid was strongly agitated for 2 to 3 minutes. The presence of saponins was confirmed by the production of froth.

Test for anthraquinones
2 mL of the solvent extract were added to 10 mL of benzene, followed by 0.5 mL of ammonia solution. The combination was cautiously shaken, and the existence of anthraquinones was confirmed by the emergence of a violet colour.

Test for phenols
2 mL of the solvent extract and 2 mL of ferric chloride solution were combined. The existence of phenols was confirmed by the production of a deep bluish-green solution.

Test for cardiac glycosides
2 ml of the solvent extract was combined with 2 ml of glacial acetic acid containing 1 drop of ferric chloride. Subsequently, 2 ml of concentrated sulfuric acid (H_2SO_4) was added. The formation of a brownish ring at the interface was taken as a symptom of the presence of cardiac glycosides.

Test for cardenolides
1 ml of the solvent extract was combined with 2 ml of benzene. The formation of a turbid brown color was considered confirmation of the presence of cardenolides.

Test for phlobtannins
A few drops of 1% hydrochloric acid (HCL) were incorporated to 1 ml of the solvent extract, which was then brought to a boil. The emergence of a crimson precipitate indicated the existence of phlobtannins.

Test for volatile oils
For this procedure, 2 ml of the solvent extract had 0.1 ml of sodium hydroxide added first, followed by a small amount of diluted hydrochloric acid. The mixture was shaken thoroughly. The formation of a white precipitate was regarded as an indication of the presence of volatile oils.

Antibacterial activity test
The procedure of well diffusion was used to assess the sample's antibacterial characteristics. A positive and a negative control were included to allow for a comparison of the outcomes.

Test organisms
The bacterial strains used in the experiment were from the Institute of Microbial Technology's (IMTECH) Microbial Type Culture Collection in Chandigarh, India.
The bacterial strains that were procured form IMTECH are listed below;
1. *Bacillus subtilis*
2. *Escherichia coli*
3. *Staphylococcus aureus*
4. *Pseudomonas aeruginosa*
5. *Aeromonas hydrophilla*

Preparation of extract solution for antibacterial activity test
All five types of the dried *Ceratophyllum demersum* extracts collected were dissolved in 10% DMSO, yielding antibacterial activity test, five different concentrations of extract solutions were used. Specifically, 4 grams of extract were dissolved in 50 ml of 10% DMSO to prepare a solution with a concentration of 100 µg/µl. Subsequently, this solution was diluted to create concentrations of 80 µg/µl, 50 µg/µl, 30 µg/µl, and 10 µg/µl. For all inoculation during the test, a fresh

stock solution of the extract was precisely prepared.

Media preparation

The composition of the nutrient agar is outlined in Table 1. For conducting the antibacterial activity test, Muller Hinton Agar (MHA) was utilized, and its composition can be referred to in Table 2. Fresh cultures were prepared using Nutrient Agar (NA) medium.

Table 1: Agar's nutritional composition

Ingredients	Amount
Peptone	0.5%
Beef extract/Yeast extract	0.3%
Agar	1.5%
NaCl	0.5%
Distilled Water	20 ml
Final pH	7.4±0.2

Table 2: Muller Hinton Agar composition

Ingredients	Amount
Beef infusion form	30%
Caesin hydrolysate	1.75%
Starch	0.15%
Agar	1.7%
pH	Neutral

Fresh Nutrient Agar Preparation

To prepare an adequate volume of this medium, a precise amount of each constituent was determined and placed in a conical flask. To attain the appropriate volume, distilled water was incorporated. The combination was subsequently heated on a Bunsen burner as long as the solution in the flask turned clear. After achieving clarity, the medium was autoclaved for 1.5 hours to ensure sterilization and removal of all impurities. Following autoclaving, the media was promptly poured into plates to prevent solidification. Sterilized plates, capable of holding 20 ml of media, were appropriately labeled before pouring the media. Once poured, the plates were allowed to solidify within the laminar air flow followed by preserved in the refrigerator for future use.

Stock culture preparation

In the antibacterial assay, five bacterial strains were utilized. Two of them were Gram-positive bacteria, namely *Staphylococcus aureus* (MTCC-2940) and *Bacillus subtilis* (MTCC-441). Additionally, three Gram-negative bacteria were employed: Escherichia coli (MTCC-739), *Pseudomonas aeruginosa* (MTCC-424), and *Aeromonas hydrophilla* (ATCC-7966). These bacterial strains were sourced from the Microbial Type Culture Collection at the Institute of Microbial Technology (IMTECH) in Chandigarh, India. To maintain the bacterial strains, sub-culturing was performed on Muller Hinton Agar (Himedia) each fifteen days, and the cultures were stored at 4 °C. Positive controls for the antibacterial assay were gentamycin discs acquired from EOS Laboratories, India. Furthermore, 10% Dimethylsulfoxide (DMSO) was employed as the negative control.

Under aseptic conditions, organisms were sub-cultured on freshly prepared Nutrient Agar (NA) plates using a sterile loop, starting from pure cultures. The pure cultures, sourced from the departmental stock's refrigerator, were thawed in the incubator for 30 minutes. Utilizing a metal loop under a laminar air flow, the organisms were streaked onto the newly prepared nutrient agar plates. These inoculated plates were appropriately labelled and incubated at 37 °C for 24 hours to facilitate optimal growth. The resulting fresh cultures were subsequently employed for the antibacterial tests (Figure 1).

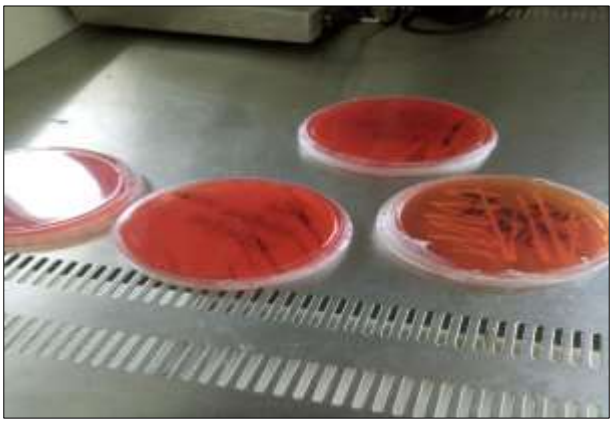

Fig 1: Fresh cultures for antibacterial tests

Test plates preparation

In order to conduct the biochemical tests, MHA was employed. A total of 2.1 grams of Muller Hinton agar was introduced into 100 ml of distilled water. This mixture was carefully stirred with a sterile spatula, heated to ensure complete dissolution of the medium, and then subjected to autoclaving for 1.5 hours for sterilization. Then, 20 ml of the prepared medium was poured into sterile plates that were appropriately labelled and left to solidify.

Inoculation of test organism:

To generate cell suspension, the test organisms were transferred via a loop into test tubes containing 5 ml of 0.9% saline. The organisms were then vortexed in a vortex machine to ensure proper mixing in the normal saline solution. 0.5 Mc farland (turbity standard) equivalent to 10^8 cfu/ml of inoculum was prepared manually by adding 0.5 ml of 0.048 M $BaCl_2.2H_2O$ to 99.5 ml of 1% H_2SO_4 with continuous stirring to retain a suspension whose density was confirmed by checking absorbance spectrophotometric ally at 625 nm. The absorbance was found in between 0.08 to 0.13. The turbidity of the standard was then compared with that of bacterial inoculums. If the bacterial inoculums appeared more turbid than the 0.5 Mc Farland, more broth was added to it. The bacteria from the cell suspension were promptly injected in the freshly produced MHA medium using a cotton swab. To ensure a regular distribution of organisms in the media, the microbial lawn was constructed by rotating them 90º each time.

Using a loop, the test organisms were placed into test tubes containing 5 ml of 0.9% saline, resulting in a cell suspension. After that, the tubes were vortexed in a vortex machine to verify that the organisms were properly mixed through the normal saline solution. A 0.5 McFarland turbidity standard, equivalent to 10^8 cfu/ml of inoculum, was manually prepared by adding 0.5 ml of 0.048 M $BaCl_2.2H_2O$ to 99.5 ml of 1% H_2SO_4, with constant stirring to maintain a suspension whose density was verified by assessing absorbance spectrophotometric ally at 625 nm. The absorbance fell within the range of 0.08 to 0.13. The turbidity of the standard was then compared with that of bacterial inoculums. If the bacterial inoculums appeared more turbid than the 0.5 McFarland standard, additional broth was added to achieve the desired turbidity. Bacteria from the cell suspension were

promptly injected into the freshly produced MHA medium using a cotton swab. The microbial lawn was generated by rotating the swab 90º each time, resulting in a homogeneous distribution of organisms throughout the medium.

Placing the extracts and controls in the plate

The plates containing the inoculated samples were appropriately labeled. Using a cork borer, wells were made in the agar. Each well received 100 µl of a stock extract, which had been previously prepared at concentrations of 100 µg/ml, 80 µg/ml, 50 µg/ml, 30 µg/ml, and 10 µg/ml using freshly prepared 10% dimethyl sulfoxide (DMSO). A positive control was established using the antibiotic Gentamycin against all bacteria, while a negative control utilized 10% freshly prepared dimethyl sulfoxide in one of the wells (Figure 2). The plates were then labelled and incubated at 37 ºC for 24 hours.

Fig 2: Positive (Gentamycin) and negative (DMSO) controls for comparison of AST results

Measuring Zones

After 24 hours of incubation for the test plates, the transparent zones were gauged using a ruler. The measurement involved determining the complete diameter of the clear zone, and these measurements were duly documented. The minimum inhibitory concentration was examined as the lowest concentration of the extract (between 10-100 mg/ml) that effectively inhibited the growth of the test microorganisms.

Results

Phytochemical analysis

Phytochemical analysis of hydrophytes revealed the presence of a variety of secondary metabolites. Both the aqueous and methanolic extracts included cardiac glycosides, steroids, phenols, flavonoids, and tannins. Table 3 shows that glycosides, anthraquinones, cardenolides, terpenoids, phlobtannins, steroids, and volatile oils were not identified in the extract.

Table 3: Initial phytochemical assessment of extracts from *Ceratophyllum demersum* using petroleum ether, acetone, ethanol, methanol, and water.

S. No.	Phytochemicals	Petroleum Ether	Acetone	Ethanol	Methanol	Water
1	Alkaloids	-	-	+	+	-
2	Anthraquinones	-	-	-	-	-
3	Glycosides	-	-	-	-	-
4	Cardenolides	-	-	-	-	-
5	Saponins	-	-	-	-	+
6	Steroids	-	-	-	-	-
7	Phlobtannins	-	-	-	-	-
8	Flavonoids	-	-	+	+	-
9	Terpenoids	-	-	-	-	-
10	Phenols	-	+	+	+	+
11	Tannins	-	-	-	-	+
12	Volatile oils	-	-	-	-	-

Note: + (present) & - (absent)

Antibacterial activity

The methanolic extract of *Ceratophyllum demersum* exhibited significant efficacy against all bacterial strains tested, displaying a zone of inhibition measuring 15 mm against *Pseudomonas aeruginosa*, 17 mm against *Escherichia coli*, 18 mm against *Staphylococcus aureus*, 18 mm against *Bacillus subtilis*, and 14 mm against *Aeromonas hydrophilla* at a concentration of 100 mg/ml. Similarly, the acetone extract demonstrated noteworthy effectiveness, showcasing a zone of inhibition measuring 15 mm against *Escherichia coli* at the same concentration of 100 mg/ml. In contrast, the negative control (10% DMSO) demonstrated no activity against any of the bacterial strains under investigation. These findings were compared to the positive control (Gentamicin), which

exhibited inhibition zones measuring 25 mm for *Bacillus subtilis* and *Pseudomonas aeruginosa*, 26 mm for *Staphylococcus aureus*, 20 mm for *Escherichia coli*, and 25 mm for *Aeromonas hydrophila* (refer to Table 4 and Figure 3).

Table 4: Zones of inhibition (in millimeter) of aqueous, petroleum ether, acetone, and ethanol, methanolic extract of *Ceratophyllum demersum* against bacterial strains

S. No.	Bacterial strain	Solvent	Concentration of *C. demersum* extract (mg/ml)					Gentamycin (10 µg/disc)
			10	30	50	80	100	
1	*Staphylococcus aureus*	Petroleum ether	-	-	-	-	-	26.20±0.00
		Acetone	-	-	-	-	-	26.20±0.00
		Ethanol	-	-	-	-	-	26.20±0.00
		Methanol	-	-	13.89^{a}±0.60	16.31^{b}±0.66	18.47^{c}±0.05	26.20^{d}±0.00
		Water	-	-	-	-	-	26.20±0.00
2	*Bacillus subtilis*	Petroleum ether	-	-	-	-	-	25.22±0.00
		Acetone	-	-	-	-	-	25.22±0.00
		Ethanol	-	-	-	-	-	25.22±0.00
		Methanol	-	11.17^{a}±0.36	14.12^{b}±0.46	16.61^{c}±0.72	18.32^{d}±0.80	25.22^{e}±0.00
		water	-	-	-	-	-	25.22±0.00
3	*Escherichia coli*	Petroleum ether	-	-	-	-	-	20.20±0.00
		Acetone	-	13.27^{a}±0.39	14.05^{b}±0.42	16.49^{c}±0.34	17.65^{d}±0.21	20.20^{d}±0.00
		Ethanol	-	-	-	-	-	20.20±0.00
		Methanol	-	11.21^{a}±0.25	12.23^{b}±0.37	15.00^{c}±0.40	16.10^{d}±0.55	20.20^{e}±0.00
		water	-	-	-	-	-	20.20±0.00
4	*Pseudomonas aeruginosa*	Petroleum ether	-	-	-	-	-	26.20±0.00
		Acetone	-	-	-	-	-	26.20±0.00
		Ethanol	-	-	-	-	-	26.20±0.00
		Methanol	-	11.39^{a}±0.81	13.25^{b}±0.39	14.62^{c}±0.80	15.02^{c}±0.50	26.20^{d}±0.00
		water	-	-	-	-	-	26.20±0.00
5	*Aeromonas hydrophilla*	Petroleum ether	-	-	-	-	-	25.02±0.00
		Acetone	-	-	-	-	-	25.02±0.00
		Ethanol	-	-	-	-	-	25.02±0.00
		Methanol	-	10.33^{a}±0.73	11.67^{b}±0.60	12.54^{b}±0.60	14.56^{c}±0.47	25.02^{d}±0.00
		water	-	-	-	-	-	25.02±0.00

Mean values with distinct superscripts within the same row indicate significant differences ($p \leq 0.05$).

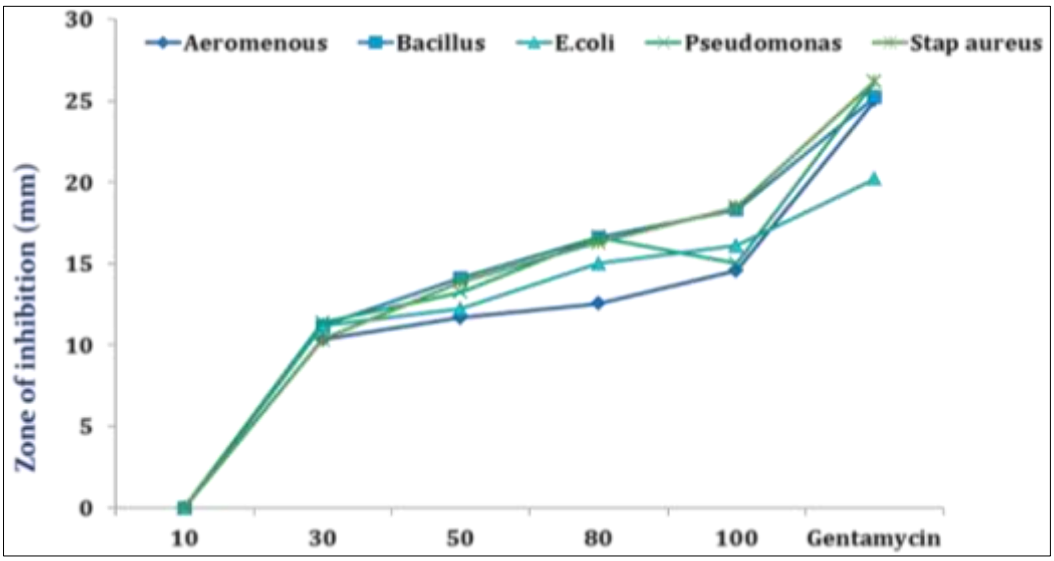

Fig 3: Zones of inhibition (in millimetre) of *Ceratophyllum demersum* extracts against bacterial strains.

Minimum inhibitory concentration (MIC)

The methanolic extract's minimum inhibitory concentration was examined to be 50 mg/ml against *Staphylococcus aureus* (Table 5). Specifically for the methanolic extract, the MIC was found to be 30 mg/ml, while it was undetectable for the remaining tested microorganisms.

Table 5: Minimum inhibitory concentration (MIC) values for aqueous, petroleum ether, ethanol, acetone, and methanolic extracts of *Ceratophyllum demersum* against the confirmed bacterial strains.

S. No.	Bacterial strain	Solvent	MIC (mg/ml)
1	*Escherichia coli* (MTCC-739)	Petroleum ether	ND
		Acetone	30
		Ethanol	ND
		Methanol	30
		Aqueous	ND
2	*Staphylococcus aureus* (MTCC-2490)	Petroleum ether	ND
		Acetone	ND
		Ethanol	ND
		Methanol	50
		Aqueous	ND
3	*Pseudomonas aeruginosa* (MTCC-2940)	Petroleum ether	ND
		Acetone	ND
		Ethanol	ND
		Methanol	30
		Aqueous	ND
4	*Bacillus subtilis* (MTCC-441)	Petroleum ether	ND
		Acetone	ND
		Ethanol	ND
		Methanol	30
		Aqueous	ND
5	*Aeromonas hydrophilla* (ATCC- 7966)	Petroleum ether	ND
		Acetone	ND
		Ethanol	ND
		Methanol	50
		Aqueous	ND

ND = not found in the measured range (10-100 mg/ml)

Discussion

The rise of drug resistance within aquaculture pathogens, along with the adverse effects linked to certain antibacterial and antifungal agents, has raised considerable concerns. This has spurred an urgent search for new antimicrobial agents [20]. The increasing resistance of bacteria to antibiotics is a critical global challenge. This escalation in bacterial resistance has reignited research into the antimicrobial properties of herbs against these resistant strains [21, 22]. Many plant leaves possess antimicrobial properties, attributed to compounds like tannins, essential oils, and various aromatic substances. Moreover, plant tannins and flavonoids have demonstrated noteworthy biological activities and antibacterial properties. Plant defence mechanisms against invading pathogens and numerous environmental stresses rely heavily on phenolic chemicals [23]. Furthermore, phenolic compounds and their derivatives, which include flavonoids, tannins, phenyl proponoids, simple phenols, lignins, and other substances, contain aromatic rings and hydroxyl groups, which influence the compound's radical scavenging capacity [24]. The present study focused on the extraction of hydrophyte *Ceratophyllum demersum* using five distinct solvents (petroleum ether, methanol, acetone, ethanol, and water). Biochemical assays were carried out to confirm the existence of several phytochemicals, encompassing alkaloids, phenols, flavonoids, tannins, steroids, saponins, and cardiac glycosides. The analysis of phytochemicals revealed a diverse array of secondary metabolites. Both the aqueous and methanolic extracts demonstrated the presence of cardiac glycosides, flavonoids, phenols, steroids, and tannins. However, terpenoids, anthraquinones, cardenolides, glycosides, steroids, phlobtannins, and volatile oils were not detected in either of the extracts. These findings align with the results reported by [25] during their phytochemical screening of various *Punica granatum* extracts. The antimicrobial effectiveness of the plant can be attributed to these phyto-constituents, which may exert their effects through various mechanisms, including disrupting the cell membrane, inhibiting cell wall formation, inactivating microbial adhesins, suppressing enzymes, or impeding nucleic acid synthesis [26]. Furthermore, research has demonstrated that phytochemical substances can disrupt the operations of multidrug-resistant (MDR) microbes by influencing their efflux pumps, resistance plasmids, beta-lactamase enzymes, and bacterial gene transposition [27]. The methanolic extract exhibited the highest activity against all bacterial strains tested, displaying a zone of inhibition measuring 16 mm against *Pseudomonas aeruginosa*, 19 mm against Escherichia coli, 16 mm against *Staphylococcus aureus*, 16 mm against *Bacillus subtilis*, and 14 mm against *Aeromonas hydrophila* at a concentration of 100 mg/ml. Similarly, the acetone extract also demonstrated significant activity, with zones of inhibition measuring 15 mm against *Escherichia coli* at the same concentration of 100 mg/ml. On the other hand, the negative control (10% DMSO) demonstrated no activity against any of the bacterial strains under examination. These findings were compared to those of the positive control (Gentamicin), which displayed inhibition zones measuring 25 mm for *Bacillus subtilis* and *Pseudomonas aeruginosa*, 26 mm for *Staphylococcus aureus*, and 20 mm for Escherichia coli. Furthermore, the methanolic extract exhibited significant activity against all tested bacterial strains, demonstrating inhibition zones measuring 15 mm against *Pseudomonas aeruginosa*, 17 mm against *Escherichia coli*, 18 mm against *Staphylococcus aureus*, 18 mm against *Bacillus subtilis*, and 14 mm against *Aeromonas hydrophila* at a concentration of 100 mg/ml. The acetone extract also displayed considerable activity, with zones of inhibition measuring 15 mm against *Escherichia coli* at the same concentration of 100 mg/ml. The negative control (10% DMSO) had no effect on any of the bacterial strains tested. In the case of *Ceratophyllum demersum*, the results were

compared to the positive control (Gentamycin), which demonstrated a zone of inhibition analysing 25 mm against *Bacillus subtilis* and *Pseudomonas aeruginosa*, 26 mm against *Staphylococcus aureus*, 20 mm against *Escherichia coli*, and 25 mm against *Aeromonas hydrophilla.* Our findings regarding the antibacterial activity of various plant extracts are consistent with prior studies, where the methanolic extract exhibited the highest activity [28, 30]. The choice of solvent used significantly affects the antimicrobial activity (AA) of an extract. This variability is likely due to differences in the solubility of various phytochemicals in distinct solvents, influenced by their relative polarities and solubilities, as reported in several studies [31, 33]. While water was traditionally employed for extraction by traditional healers, investigation indicates that the extracting solvent plays an important role when analysing the pharmaceutical properties of a medicinal plant [34]. Concerning microbial susceptibility, *Escherichia coli* and *Staphylococcus aureus* were the most susceptible bacterial strains. Similar findings were obtained by [35] in their study on the antibacterial activity of different solvent extracts of *Melia azedarach*. The minimum inhibitory concentration of the methanolic extract against *Staphylococcus aureus* in *Ceratophyllum demersum* plants was determined to be 50 mg/ml. However, the MIC for the methanolic extract was detectable only at a concentration of 30 mg/ml, remaining undetected in the rest of the test microbes.

Conclusion

The rising drug resistance in aquaculture pathogens and the detrimental effects associated with specific antibacterial and antifungal drugs emphasize the critical necessity for novel antimicrobial agents on a worldwide level. Plant leaves, rich in antimicrobial compounds like tannins and flavonoids, offer a promising avenue. This study was devoted to extracting hydrophytic *Ceratophyllum demersum* using various solvents and conducting thorough phytochemical analysis. The methanolic extract demonstrated the highest antibacterial activity against the strains tested, underscoring the pivotal role of solvent choice in influencing antimicrobial efficacy. There is a growing interest in utilizing natural hydrophytic plant extracts as antimicrobials, particularly to combat the surge in foodborne diseases and reduce reliance on synthetic drugs. The findings of this study highlight the potential of *Ceratophyllum demersum* as a natural remedy for infectious diseases in aquaculture, showing promise against a spectrum of bacteria and aquaculture-related pathogens.

Acknowledgement

The authors are thankful to the Dean Faculty of Fisheries, SKUAST-Kashmir, Rangil (J&K) for the study's necessary facilities.

References

1. De Paola A, Peeler JT, Rodrick G. Effect of oxytetracycline-medicated feed on antibiotic resistance of Gram-negative bacteria in catfish ponds. Applied and Environmental Microbiology. 1995;61:2335-2340.
2. Abutbul S, Golan-Goldhirsh A, Barazani O, Ofir R, Zilberg D. Screening of desert plants for use against bacterial pathogens in fish. Isr. J Aquacult.-Bamid. 2005;57(2):71-80.
3. Direkbusarakom S. Application of medicinal herbs to aquaculture in Asia. Walailak Journal of Science and Technology. 2004;1(1):7-14.
4. Rios JL, Recio MC. Medicinal plants and antimicrobial activity. J Ethnopharmacol. 2005;100:80-84.
5. Punitha SMJ, Babu MM, Sivaram V, Shankar VS, Dhas SA, Mahesh TC, *et al*. Immunostimulating influence of herbal biomedicines on nonspecific immunity in Grouper *Epinephelus tauvina* juvenile against *Vibrio harveyi* infection. Aquaculture international. 2008;16:511-523.
6. Austin B, Austin DA. Bacterial Fish Pathogens: Disease of Farmed and Wild Fish. Praxis Publishing, UK; c2007. p. 581.
7. Pratt R, Daniel TC, Gunnison JB, Kumler WD, Oneto JF, Strait LA *et al*. Chlorellin: An antibacterial substances from Chlorella, Science. 1944;99:351-352.
8. Hernández-Ledesma B, Herrero M. Bioactive compounds from marine foods: plant and animal sources. John Wiley & Sons; c2013.
9. Dixit S, Vishnoi N, Singh DP. Algae as a Source for Nutraceutical Products: Scope and Perspective. In Algae and Sustainable Technologies. CRC Press; c2020. p. 245-270.
10. Mondo DA, Smerilli A, Ambrosino L, Albini A, Noonan DM, Sansone C, *et al*. Insights into phenolic compounds from microalgae: Structural variety and complex beneficial activities from health to nutraceutics. Critical Reviews in Biotechnology. 2021;41(2):155-171.
11. Kubanek J, Jensen P, Keife RPA, Cameron MS, Collins DO, Fenical W. Seaweed resistance to microbial attack: A targeted chemical defense against marine fungi. PNAS. 2003;100:6916-6921.
12. Bansemir A, Blume M, Schröder S, Lindequist U. Screening of cultivated seaweeds for antibacterial activity against fish pathogenic bacteria. Aquaculture. 2006;252:79-84.
13. Kolanjinathan K, Ganesh P, Govidarajan M. Antibacterial activity of ethanol extracts of seaweeds against fish bacterial pathogens. European review for medical and pharmacological sciences. 2009;13:173-177.
14. Kumar GS, Sarada DVL, Rengasamy R. Seaweed extracts control the leaf spot disease of the medicinal plant *Gymnema sylvestre*. Indian Journal of Science and Technology. 2008;1(3):1-5.
15. Engel S, Puglisi MP, Jensen PR, Fenical W. Antimicrobial activities of extracts from tropical Atlantic marine plants against marine pathogens and saprophytes. Marine Biology. 2006;149:991-1002.
16. Martinez-Nadal NG, Rodriguez LC, Casillas C. Sarganin and Chonalgin new antibiotic substances from marine algae Puerto Rico. Antimicrb. Agents. Chemother; c1963. p. 68-72.
17. Caccamese S, Azzolina R, Furnari G, Cormaxi M, Grasso S. Antimicrobial and antiviral activities of extracts from Mediterranean algae. Botanica Marina. 1980;23:285-288.
18. Blunt JW, Copp BR, Hu WP, Munro MHG, Northcote PT, Prinsep MR, *et al*. Marine natural products. Natural Product Reports. 2007;24:31-86.
19. Harborne JB. Phytochemicals method. London. Chapman and Hill; c1973.
20. Phongpaichit S, Subhadhirasakul S, Wattanapiromsakul C. Antifungal activities of extracts from Thai medicinal plants against opportunistic fungal pathogens associated with AIDS patients. Mycoses. 2005;48:333-338.
21. Hemaiswarya S, Kruthiventi AK, Doble M. Synergism between natural products and antibiotics against

infectious diseases. Phytomedicine. 2008;15:639-652.
22. Alviano DS, Alviano CS. Plant extracts: search for new alternatives to treat microbial diseases. Curr. Pharm. Biotechnol. 2009;10:106-121.
23. Hellio C, La DB, Dufosse D, Le G, Bourgougnon N. Inhibition of marine bacteria by extracts of macroalgae: potential use for environmentally friendly antifouling paints. Mar Environ. Res. 2001;52(3):231-247.
24. Dziedzic SZ, Hudson BJF. Hydroxyisoflavones as antioxidants for edible oils, Food Chem. 1983;11:161-166.
25. Belkacem N, Djaziri R, Lahfa F, El-Haci IA, Boucherit Z. Phytochemical screening and *in vitro* antioxidant activity of various *Punica granatum* peel extracts from Algeria: A comparative study. Phytothér. 2014;12:372-379.
26. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev. 1999;12:564-582.
27. Mahindra R, Kateryna K. (1st ed.), Fighting multidrug resistance with herbal extracts essential oils and their components. Academic press, Elsevier; c2013.
28. Kavishankar GB, Lakshmidevi N, Mahadeva MS. Phytochemical analysis and antimicrobial properties of selected medicinal plants against bacteria associated with diabetic patient, International Journal of Pharmaceutical and Bio Science. 2011;2:509-518.
29. Swamy MK, Sudipta KM, Lokesh P, Neeki AM, Rashmi W, Bhaumik HS, *et al*. Phytochemical screening and *in vitro* antimicrobial activity of *Bougainvillea spectabilis* flower extracts, International Journal of Phytomedicine. 2012;4:375-379.
30. Wani SA, Shah KW, Ahmad MA. Antifungal activities of methanolic extracts of *Podophylum hexandrum* and *Rheum emodi* against human pathogenic fungal strains, International Journal of Pharmaceutical Sciences Review and Research. 2013;19(2):56-59.
31. Al-Zubaydi SR, Al-Hmdany MA, Raesan SJ. Anti-bacterial effect of some medicinal plant extracts against some pathogenic bacteria strains. J Duhok Univ. 2009;12:244-249.
32. Boklari FM Antifungal activity of some medicinal plants used in Jeddah, Saudi Arabia. Mycopathologia. 2009;7:51-57.
33. Bakht J, Muhammad T, Ali H, Islam A, Shafi M. Effect of different solvent extracted sample of *Allium sativum* (Linn) on bacteria and fungi. African Journal of Biotechnology. 2011;10(31):5910-5915.
34. Parekh J, Jadeja D, Chanda S. Efficacy of aqueous and methanolextracts of some medicinal plants for potential antibacterial activity. Turk J Biol. 2005;29:203-210.
35. Antara S, Amla B. Evaluation of antimicrobial activity of different solvent extracts of medicinal plant *Melia azedarach* L. Int J Curr Pharmaceutl Res. 2005;4:67-73.