Antibacterial Effect Of *Punica Granatum* Acetone Leaf Extract Against *Aeromonas Hydrophila* Affecting Gold Fish *Carassius Auratus*

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ABSTRACT

In the present study, *Aeromonas hydrophila*, a bacteria isolated from infected goldfish (*Carassius auratus*), was tested for its antibacterial efficacy against *Punica granatum* leaf - acetone extract. Diffusion disc plates on agar were used to measure the antibacterial activity. Different concentrations (25 %, 50 %, 75 % and 100 %) of *P. granatum* leaf - acetone extract was tested against *A. hydrophila.* The *P. granatum* leaf – acetone extract was exhibited a significant level of inhibition against *A. hydrophila*. Maximum zone of clearance was measured after 24 hours of incubation at 37°C. The result of the present study revealed that the fish pathogen *A. hydrophila* was exhibited maximum zone of clearance (18 mm) at 100 % concentration of *P.granatum* leaf - acetone extract, where as the minimum zone of clearance 8 mm was recorded at 25 % of concentration. Fourier Transform Infra-Red Spectroscopy (FTIR) of *P. granatum* leaf –acetone extract revealed the presence aliphatic, alkenes, nitrogen, carbonyl and hydroxyl groups. The phytochemical analysis of the *P. granatum* leaf – acetone extract showed the presence of tannins, carbohydrates, terpenoids, phenols, proteins and steroids. As per the result of the present study, it can be concluded that phytochemicals were identified in *P. granatum* leaf – acetone extract was effective against *A. hydrophila*, a microbial pathogen that has infected goldfish (*C.auratus*).

Keywords: *A. hydrophila, P. granatum*, extracts, antibacterial activity, FTIR analysis.

1. INTRODUCTION

The fish pathogen *A. hydrophila* is one of the most prevalent bacteria in freshwater environments globally [1]. *Aeromonas* sp. is a Gram-negative, Aeromonadaceae - family bacteria that are facultative pathogens. Diverse bacterial species of *Aeromonas* sp. is responsible for the significant mortality of infected wild and farmed fishes, inflicting enormous economic damage [2]. It causes several zoonotic illnesses in human, fish and also linked to cellulitis, an infection that produces tissue inflammation [3, 4]. *Aeromonas* sp. causes diseases called gastroenteritis [5], an inflammation of the respiratory system, and diarrhoea followed by fever [6]. Sepsis is a deadly consequence of infections caused by *Aeromonas* sp. It has been identified as the most prevalent pathogenic bacterial species in aquaculture and is often responsible for lesions on fish farms and high death rates, which have provoked a global economic catastrophe in the aquaculture sector [7, 8].

In addition to affecting the microbiota of aquatic habitats, the unregulated use of antibiotics to suppress pathogenic microbes also resulted in inappropriate changes to the microbiota of these systems [9,10]. Humans who routinely consume antibiotic-treated fish have developed serious health issues [11]. Additionally, fishes were produced immune system with damaged antibodies which works against specific, adaptive pathogenic bacteria [11, 12]. At present, numerous fish diseases are treated by using plant-based extracts in order to increase innate behaviour and

immunity, as well as to prevent the spread of infection [13, 14,15]. Attempts have been made to use plant-based extracts to combat fish infections as a substitute for commercial medicines that protect certain pathogenic bacteria from resistance [16, 17]. According to Santos *et al.*, (1995) a lot of medications have been identified in recent years from medicinal plants [18]. In industrialized nations, 80% of the population uses traditional medicines derived from plants, which act an effective therapeutic agent in both contemporary and traditional medical systems [19].

Since ancient times, pomegranates (*P. granatum L.*), a member of the Punicaceae family, have attracted international attention [20]. This plant and fruit are revered from ancient times, very adaptable to an extensive range of soil and temperature conditions and cultivated in a wide range of geographic regions including the California, Mediterranean basin and Asia. Current research confirms that pomegranate leaf, fruit, bark and flower contain bioactive phytochemicals that are responsible for antimicrobial activity and prevent life-threatening diseases [21]. This study investigated the antibacterial activity of *P. granatum* leaf – acetone extract against *A. hydrophila* infected goldfish (*C. auratus*).

2. MATERIALS AND METHODS

2. 1 Collection of *P. granatum* plant leaves

Fresh leaves of *P. granatum* were collected from Kanyakumari District of Tamil Nadu, India and transported to laboratory. The leaves were rinsed with distilled water before being dried in the shade under hot air with a maximum temperature of 40°C. The samples were adequately crushed using a mortar and pestle to obtained a fine, homogenous powder, which was then stored in paper bags free from moisture [22].

2.2 Preparation of plant extracts

P. granatum leaf extracts were obtained utilize a continuous extraction system (Soxhlet extractor) using the method given by Wang (2006) [23] for extracting plant leaves with organic solvents such as acetone: In addition to 300 mL of acetone, 30 g of plant powder was added to the thimble holder of the Soxhlet device (rate 1:10 w: v). In the thimble-holder of Soxhlet apparatus, a 75% acetone extraction solvent was utilized. Four hours were spent extracting until the solvent that emerged from the thimble turned colourless. Subsequently, in order to concentrate the extracts, they were dried using a rotating vacuum evaporator at temperatures below 40 ° C until the moisture content reached around 8 % (Dry basis). The crude extracts were filtered to obtain by using Whatman No. 1 filter paper. Samples were transferred in sterile vials and refrigerated at 4 ° C for future studies.

2.3 Phytochemical analysis of *P. granatum* leaf-acetone extract

The phytochemical analysis of crude *P. granatum* leaf-acetone extracts was conducted to determine the presence or absence of various bioactive constituents or secondary metabolites such as carbohydrates, coumarins, tannins, saponins, flavonoids, glycosides, phenols, proteins, steroids, and terpenoids using standard protocols [24].

2.4 Chromatographic purification of *P. granatum* leaf extract

2.4.1 Thin Layer Chromatography of *P. granatum* leaf extract

Thin-Layer Chromatography (TLC) using petroleum ether: acetone (3:1) solvent systems were used to identify the primary constituents contained in the *P. granatum* leaf – acetone extract.

The chamber is composed of a petroleum ether: acetone solvent, and the extract is delivered via capillary tubes on a TLC plate that has been pre-coated. To determine the spot of each applied leaves extract on the plate, a thin line and dots are drawn on the plate. After each mobile phase was applied to the TLC plate, it was air-dried and examined under ultraviolet light. The development of the separated bands as reflected by their retention factor (R_f) values was determined [25].

2.5 FTIR (Fourier transform infrared spectroscopy) analysis of *P. granatum* leaf – acetone extract

Using Fourier transform infrared (Bruker, Alpha T, Germany), the distinctive functional groups in the *P. granatum* leaf – acetone extract was identified. It is often possible to get clear information about the structure of a molecule from its absorption spectrum. A small amount of extract from *P. granatum* leaves was combined with dry potassium bromide (KBr). Mixing the sample with potassium bromide in a mortar and compressed it at a pressure of 6 bar produced the disc. The disc was then put in a sample cup of a diffuse reflectance accessory. The IR spectrum was acquired using an infrared spectrometer. The specimen was scanned between 4000 and 400 cm^{-1}. The FTIR peak values were recorded [26].

2.6 Collection of naturally infected goldfish - *C. auratus*

The infected gold fish sample was gathered from an aqua farm at Kanyakumari District of Tamil Nadu, India. Various kinds of clinical signs and behavioral alterations have been discovered and characterized in these infected gold fish. Under a microscope, scrapings from the body surface and fins of diseased gold fish were examined to detect and characterize the presence of moulds and other parasites [27].

The samples were placed in sterile tubes with strap closures, sample name, collection location, and collection date were recorded on the card number.

2.7 Isolation of bacterial strains from infected goldfish (*C. auratus)*

Naturally infected gold fish were dissected and the infected epidermis was homogenized with sterile PBS (Phosphate Buffer Saline). The sample was serially diluted to reduce bacterial proliferation [28]. The sample was then inoculated into nutrient agar using the spread plate method [29]. After examining and counting the colonies, the plates were incubated for 24 hours at 37^0C. On the basis of physical similarities, colonies were chosen and streaked on nutrient agar media until a pure culture was obtained. Pure colonies were picked up and streaked on nutrient agar slants and stored at 4°C for subsequent identification.

2.8 Characterization and identification of pathogens

A. hydrophila was identified by its colony form, gram nature, shape and motility. In addition, the isolates were subjected to the following biochemical tests to identify the bacteria based on their reactions: Indole, methyl red, Voges-Proskauer, citrate utilization, nitrate reduction, hydrogen sulphide production, urease, catalase and oxidase. The outcomes of biochemical characterization were compared to those published in earlier studies [28].

2.8.1 16s rRNA sequencing - molecular identification of bacterial strains.

The morphological, biological, physiological and biochemical characterization of the bacterial strains can be identified from the infected gold fish. According to Bergey's Manual of Determinative Bacteriology, the biochemical characterization of bacterial isolates was performed. The 16s rRNA sequencing of bacterial strains was used to confirm their pathogen classification. Using the phenol: chloroform method, the genomic DNA was extracted and purified [30].

2.9 Antibacterial activity of *P. granatum* leaf – acetone extract

Different concentrations of the extracts (25 %, 50 %, 75 % and 100 %) were tested against *A. hydrophila*. In order to cultivate the bacterial strain, nutrient agar was used. Agar-based diffusion disc plates were utilized to assess the antibacterial activity [31]. The antibiotic disc was commercially available. The antibiotic disc were used as the positive control and placed on the centre of the agar plates.

Added different concentrations of leaf extracts of *P. granatum* (25 %, 50 %, 75 %

and 100 %) on each well. On the agar plate, the zones of inhibition (diameter in mm) were examined after each plate was incubated at 37°C for 24 hours. The antibacterial activity of solvent blanks produced in the same manner was examined [32].

3. RESULTS

3.1 Identification of pathogens and biochemical analysis

The isolated bacteria from infected goldfish (*C. auratus*) were identified as *A. hydrophila* based on their biochemical and morphological characterization. The 16S rRNA gene sequencing further confirmed as *A. hydrophila.*

3.2 Qualitative analysis of phytochemical analysis of *P. Granatum* leaf -acetone extract

The phytochemical analysis indicated the presence of bioactive chemical compounds such as coumarins, tannins, saponins, flavonoids, glycosides, phenols, proteins, steroids, terpenoids, as well as carbohydrates. Table 1 shows the results of a qualitative phytochemical analysis of *P. granatum* leaf – acetone extract. The phytochemical analysis of *P. granatum* leaf – acetone extract showed the presence of tannins, carbohydrates, terpenoids, phenols, proteins and steroids (Plate.1).

Plate 1: Phytochemical analysis of *P. granatum* leaf – acetone extract

Sl. No.	Phytochemical screening	Acetone
1	Tannins	Positive
2	Flavonoids	Negative
3	Carbohydrates	Positive
4	Terpenoids	Positive
5	Phenols	Positive
6	Proteins	Positive
7	Steroids	Positive
8	Saponins	Negative
9	Coumarins	Negative
10	Glycosides	Negative

Table 1: Phytochemical constituents of *P. granatum* leaf – acetone extract

3.3 Thin Layer Chromatography of *P. granatum* leaf – acetone extract

Thin Layer Chromatography (TLC) was utilized using petroleum ether: acetone (3:1) solvent systems in order to identify the primary components that were found in the most effective extracts of *P. granatum* leaves. Retention factor (R_f) values were obtained to express the movement of the separated bands (Plate .2).

$$RF = \frac{\text{Distance Traveled by the Compound}}{\text{Distance Traveled by the Solvent Front}}$$

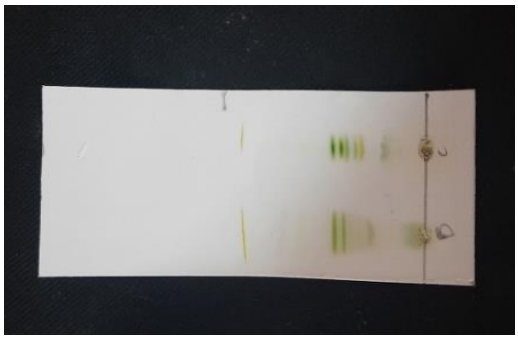

Plate2: Thin Layer Chromatography analysis of *P. granatum* leaf – acetone extract

Sl. No.	Color of spot	R_f value of sample	R_f value of control	Pigment
1.	Greenish yellow	0.08	NB	Viola xanthine
2.	Grey	0.3	NB	Unidentified
3.	Light green	0.42	0.43	Chlorophyll b
4.	Green	0.46	0.46	Chlorophyll a
5.	Dark yellow	0.9	0.95	B - carotene

Table 2: Identifying the pigment by comparing the R_f value of sample and control

Thin-layer chromatography can be used to separate and distinguish the pigments in leaf extract (Table.2). Chlorophylls, which are green pigments that act as the primary photoreceptor molecules in plants. Yellow pigments known as carotenoids aid photosynthesis in plants. Additionally, chloroplasts include xanthophylls, which may be separated and analyzed via chromatographic methods.

3.4 FTIR Spectrum of *P. granatum* leaf – acetone extract

The FTIR spectrum was performed to determine the functional groups of the bioactive constituents in *P. granatum* leaf – acetone extract based on the maximum value in the region of infrared radiation.

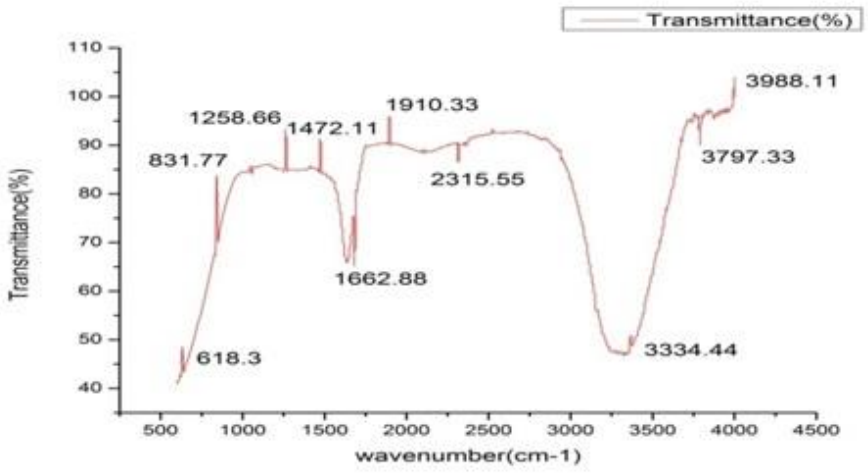

Plate3: FTIR Spectrum of *P.granatum* leaf - acetone extract

The results of the FTIR analysis of *P. granatum* leaf - acetone extract showed in Plate. 3. The graph shows the distribution of different functional groups within the sample. Plate.3 indicates that an FTIR evaluation of *P. granatum* leaf - acetone extract was carried out in the 500–4500 cm^{-1} range of wave number. The graphs show the existence of different chemical groups, and FTIR signals are produced from corresponding C=O vibrations of oxygen (618.3 $cm^{-1)}$, C=C stretching of alkenes (831.77 0 cm^{-1}), then (1258.66 cm^{-1}) C=C stretching of alkenes, (1472.11 cm^{-1}) N-H stretching of nitrogen groups, (1662.88 cm^{-1}) N-H stretching of aliphatic groups,(1910.33 cm^{-1}) C=O carbonyl group,(2315.55 cm^{-1}) O-H hydroxyl group, (3334.44 cm^{-1}) hydroxyl group,(3797.33 cm^{-1}) C=O carbonyl group,(3988.11 cm^{-1}) C=C alkenes group.

3.5 Antibacterial Activity of *P. granatum* leaf – acetone extract against *A. hydrophila*

The antibacterial activity of samples was evaluated against the identified strains of bacteria. The inhibition zone of sample extract was varying depending on the microorganism and the solvent used for the extraction. The zone of inhibition indicated that the action of these samples against the bacteria. The antimicrobial activity of the *P. granatum* leaf - acetone extract samples was initially evaluated by agar plate diffusion method using *A. hydrophila* isolated from infected goldfish (*C. auratus).*

Sample	Solvent used	ZONE OF INHIBITION					
		Concentration leaves extract				Positive control (mm)	Negative Control DMSO (mm)
		0.25µl (25%)	0.5 µl (50%)	0.75 µl (75%)	1.0 µl (100%)		
P.granatum	Acetone	8mm	10mm	15mm	18mm	21mm Amoxicillin	No Zone

Table 3: Antibacterial activity of different concentrations of *P. granatum* leaf – acetone extract

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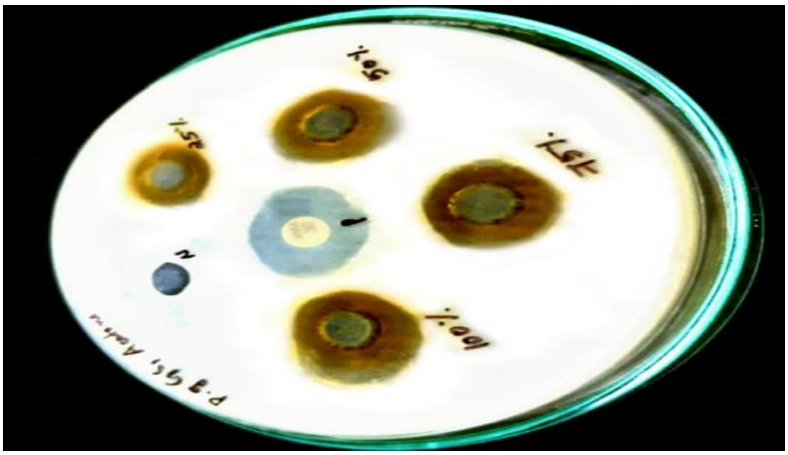

Plate 4: Antibacterial activity of different concentrations of *P. granatum* leaf – acetone extract

The efficacy of acetone extract to inhibit the in vitro growth of *A. hydrophila* was showed in Table 3. The acetone extracts of *P. granatum* leaves were tested with four different concentrations of 25 %, 50 %, 75 % and 100% respectively, against the fish

pathogens of *A. hydrophila* (Plate 4). In the present study, *P. granatum leaf - acetone extract* leaves showed maximum zone of inhibition at the concentration of 100% (18mm). Whereas the 50 % and 75 % of *P. granatum* leaf – acetone extract resulted the moderate zone of inhibition of 10 mm, 15mm respectively.

4. DISCUSSION

In India, a substantial amount of research has been conducted on traditional system of medicinal plants, since there has been a recent surge in interest in a wide range of traditional natural products. As an alternative to the use of antibiotics in aquaculture, the use of natural and harmless substances holds promise. Aquaculture involves use of a wide variety of medicinal plants [36]. More than 60 different medicinal plant species have been investigated to improve fish health and disease prevention in aquaculture. [37]. To demonstrate the effectiveness of herbs in aquaculture as effective treatments for bacterial infections in aquatic animals, further research on herbs is required [38]. According to many researchers, *P. granatum* plants have antibacterial and other properties. [33,39]. To address therapeutic requirements, many researchers have examined the anti-inflammatory and antioxidant effects of drugs derived from *P. granatum* [40]. For the therapy of a wide spectrum of human diseases, especially bacterial infections, almost every component of the pomegranate tree was employed in naturally derived medicine [32]. The extraction solvent (aqueous, ethanolic, acetone, chloroform, etc.) alters the range of antibacterial activity. The crude extract has stronger antibacterial activity than the fragmented extract because the antibacterial activity is increased by the synergistic effect of the diverse chemical components, some of which may be lost during fractionation [37]. Phytochemical extract of pomegranate plant have the potential to be sources of antibacterial substances. [34,35]. The phytochemical analysis of pomegranate leaf - acetone extracts in this study indicated the presence of various components including tannins, carbohydrates, terpenoids, phenols, proteins, and steroids. The supporting prior findings, who observed the presence of identical phytoconstituents in different extracts of *P. granatum* leaf extract [22]. The results of *P. granatum* leaves FTIR analysis confirmed the presence of alkanes, carbonyl group, hydroxyl group, nitrogen groups which shows major peaks at 618.3 $cm^{-1}$for C=O vibrations of oxygen, 831.77 0 cm^{-1} for C=C stretching of alkenes, 1258.66 cm^{-1} for C=C stretching of alkenes, 1472.11 cm^{-1} for N-H stretching of nitrogen groups, 1662.88 cm^{-1} for N-H stretching of aliphatic groups,1910.33 cm^{-1} for C=O carbonyl group, 2315.55 cm^{-1} for O-H hydroxyl group, 3334.44 cm^{-1} for hydroxyl group, 3797.33 cm^{-1} for C=O carbonyl group, 3988.11 cm^{-1} for C=C alkenes group. The extract of pomegranate leaves has been shown by some prior researches to have antibacterial activity against both gram-negative and gram-positive bacteria (biofilm and planktonic), viruses, fungi and parasites [41]. The bacteria *M. luteus, C. xerosis, P. aeruginosa, S. aureus, E. coli, E. faecalis and B. megaterium* were shown to be resistant to the extracts taken from the fruits of six common pomegranate cultivars[42]. The antibacterial activity of pomegranate rind extracts (aqueous and alcoholic and aqueous) against a variety of enteric pathogens, including *Vibrio cholerae,* Enteropathogenic *Escherichia coli* (EPEC), Enterotoxigenic *Escherichia coli*, Enteroaggregative *Escherichia coli*, *Salmonella* and *Shigella* species, along with a few strains of *Candida* [43]. In this study, the antibacterial activities *P. granatum* might be attributed to the tannins, flavonoids, and phenolic acids that can be detected in the leaf extracts [43]. In comparison to the results of previous studies, the outcome of the present study showed *A. hydrophila* isolates were observed in infected goldfish (*C. auratus)* showed a promising zone of inhibitions from leaves acetone extract of 100 % (18 mm) followed by 75 % (15 mm), 50 % (10 mm), and 25% (8 mm), respectively. The acetone extract from *P. granatum* leaves can be used as a substitute treatment for

bacterial strains that cause fish disease. This study reveals that the *P. granatum* leaf acetone extract is effective in vitro against *A. hydrophila*. Treatment with an extract of *P. granatum* leaves seems most promising for the treatment of *A. hydrophila* infection in *C. auratus* gold fish.

5. CONCLUSION

The medicinal plants continue to be important therapeutic agents in both conventional and modern healthcare systems. The plant derived extracts are utilized to treat a variety of fish ailments in order to promote natural behavior and immunity as well as to prevent the transmission of infection. *P. granatum* is a remarkable medicinal herb with a wide range of therapeutic characteristics, according to phytochemical analysis of its leaves. In this study, *A. hydrophila*, a bacterium isolated from infected goldfish (*C. auratus*) was resistant to *P. granatum* leaf – acetone extract, which shown promising antibacterial activity.

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