

EXTRACTION AND CHARACTERIZATION OF POMEGRANATE PEEL AS IMMUNOSTIMULANT FOR AQUACULTURE APPLICATION

PROJECT WORK

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Science degree in Microbiology

By

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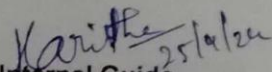
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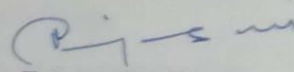
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This is to certify that this Project Report is the bonafide work of LAKSHMI PRIYA S (41300020) who carried out the project "EXTRACTION AND CHARACTERIZATION OF POMEGRANATE PEEL AS IMMUNOSTIMULANT FOR AQUACULTURE APPLICATION" under our supervision from January 2024 to April 2024.


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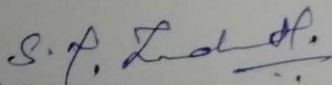
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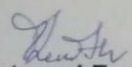


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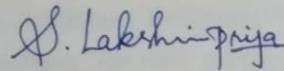

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DECLARATION

I, LAKSHMI PRIYA S (41300020) hereby declare that the Project report entitled "EXTRACTION AND CHARACTERIZATION OF POMEGRANATE PEEL AS IMMUNOSTIMULANT FOR AQUACULTURE APPLICATION" done by me under the guidance of Ms. S. HARITHA (Internal) and Dr. M. V. RAJESHWARI (External) at CENTRE FOR AQUACULTURE, SATHYABAMA RESEARCH PARK, SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY, is submitted in partial fulfilment of the requirements for the award of a Bachelor of Science degree in Microbiology.

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ABSTRACT

Aquaculture, is a rapidly growing industry, faces challenges related to disease outbreaks that can significantly impact production. In the quest for sustainable and natural alternatives to enhance the immune response of aquatic organisms, this study explores the bioactive components present in the pomegranate peel as an immunostimulant in the aquaculture application. Pomegranate peel, a by product of the fruit processing industry, is rich in bioactive compounds known for their antioxidant and immunomodulatory properties. The maximize the yield of immunostimulatory compounds from the pomegranate peel was extracted by ethanol. Characterization of the extracted compounds is performed using analytical techniques, including GCMS and FT-IR, to identify and quantify specific bioactive constituents. The extracted compound performed antibacterial and immunostimulant screening against aquatic pathogens such as *Aeromonas hydrophila*, *Aeromans caviae*, *Edwardsiella tarda*, and *Staphylococcus aureus* respectively. Experimental fish diets were prepared and supplemented to the fish. Results revealed that the growth performance of fish fed the experimental diets was decreased. The results showed that even at low concentrations (50 µg) of pomegranate peel extract showed a potent antibacterial effect against *A. hydrophila*, *A. caviae*, *E. tarda*, and *S. aureus*. The study aims to provide valuable insights into the development of a natural and sustainable immunostimulant for aquaculture, contributing to the overall health and productivity of aquatic organisms while minimizing the reliance on synthetic additives.

Keywords: Pomegranate peel, Immunostimulant, Bioactive components

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LIST OF ABBREVIATIONS

CAGR	compound annual growth rate
ALT	alanine aminotransferase
AST	aspartate aminotransferase
GC-MS	Gas chromatography mass spectroscopy
FTIR	Fourier transform infrared spectroscopy
SGR	specific growth rate
ANOVA	Analysis of variance
MIC	minimum inhibitory concentration
MBC	minimum bactericidal concentration
ADG	average daily gain
FCR	Feed conversion ratio
PPE	Pomegranate peel extract

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CHAPTER 1

INTRODUCTION

1.1 AQUACULTURE

Global production of fish from aquaculture has grown rapidly over the past four decades, contributing significant quantities to the world's supply of fish for human consumption. Aquaculture now accounts for almost half (45%) of the world's food fish (FAO 2006a). Global aquaculture production of aquatic animals has been growing at an average rate of 5.3% in the period 2001–2018 (FAO, 2020). In 2018, the aquaculture industry had produced around 82 million tons globally, which is expected to reach 109 million tons by 2030. On average, the growth rate for aquaculture production from 2001 to 2018 was around 5.3%. Asia is the major contributor with 89% of the global aquaculture production. The main products from aquaculture are finfish (54.3 million tons) followed by molluscs (17.7 million tons), crustaceans (9.4 million tons), marine invertebrates (435,400 tons), aquatic turtles (370,000 tons), and frogs (131,300 tons) (FAO, 2020).

Aquaculture production (fish and shellfish only) in the leading producer countries in the world: China (about 58% of global production), India (8.6%), Indonesia (6.6%), Vietnam (5%) and Bangladesh (about 3%). Of these, only Indonesia does not exceed 50% of aquaculture products of the total (capture fisheries and aquaculture) production, accounting for 43% of farmed products. (FAO 2020). The growth of aquaculture production also has positive impacts in terms of employment generation. Approximately 23 million full-time jobs, either directly or indirectly and especially in developing countries, are linked to aquaculture (Nasr-Allah *et al.*, 2020; Ottinger *et al.*, 2016).

1.2 ORNAMENTAL FISH

Ornamental fishes, due to their different and brilliant colors, shapes and behavior, are often referred to as living jewels and are kept in aquaria or garden pools for their beauty as well as entertainment. Ornamental fishes, thanks to their different and brilliant colors, shape and behavior, are often referred to as living jewels and are kept in aquaria or garden pools for entertaining and fancy, thus resulting, together with photography, among the most important hobbies worldwide. Owning and photography of ornamental fishes are popular hobbies worldwide. There is an increase in people who are more inclined towards purchasing attractive fish species for decorative purposes, citing their alluring features and differing characteristics. This has fueled the exponential growth of the ornamental fish market (Kumar *et al.*, 2015). Nowadays, more than 7000 aquatic species are reared and marketed as ornamental fish. Of them, approximately 5000 are freshwater- and 1800 are marine species (Chen *et al.*, 2020). More than 120 countries are involved in the ornamental fish industry, in their import/export, led by Asian and developing countries, which produce approximately 60% of ornamental fish (Evers *et al.*, 2019), with a global trade worth approximately of USD 15–30 billion each year (Biondo and Burki 2019).

The ornamental fish market was valued at USD 5.4 billion in 2021 and is anticipated to expand at a compound annual growth rate (CAGR) of 8.5% from 2022 to 2030. The tropical freshwater fish segment dominates the market and accounted for the largest revenue share of 51.7% in 2021. Among most popular tropical freshwater fish for beginners are guppy (*Poecilia reticulata*), molly (*Poecilia sphenops*), and zebrafish (*Danio rerio*); most of them are quite inexpensive and usually priced between USD 1 and 6. Nearly 15% of the total traded fish consists of marine ornamental species, with their attractive colors and attention-grabbing behaviour (Raja *et al.*, 2014) largely derived from wild catches (Satam *et al.*, 2018). Approximately 99% of the market is kept by hobbyists and less than 1% by public aquaria and research institutes. It has been estimated that more than 1.5 million people are involved in this sector and there are over 3.5 million hobbyists

worldwide involved in this trade. The sector has provided employment opportunities, alleviated poverty, and contributed to national income by enhancing foreign exchange earnings (Dey 2010).

1.2.1 Koi carp (*Cyprinus carpio*)

Koi carp (*Cyprinus carpio*) are high-value ornamental fish and are in great demand due to their beauty, colour, and attractive body shape; furthermore, koi lovers in Indonesia believe that they bring good luck to their owners (Kusrini *et al.*, 2015). Quality strongly influences the value and demand for koi carp as ornamental fish. In addition to good health without any defects, the beauty of the body shape, and the colouration including the pattern and colour intensity are also important factors for all ornamental fishes, while growth rates affect the profitability of ornamental fish businesses (Zulfikar *et al.*, 2018). The colours that are usually found in koi carp are red, white, yellow and black. As colour is such an important component of the beauty of these ornamental fish, cultivators need to be able to maintain and improve the colour quality of koi carp. One possible way to improve the colour quality of ornamental fish is by rearing them in tanks with certain background colours (Harini *et al.*, 2019, Yasir and Qin, 2009).

1.3 CURRENT PROBLEM FACED IN AQUACULTURE INDUSTRY

Aquaculture is growing more rapidly than any other animal-production sector. Its relative contribution to the total amount of fish produced for human consumption changed from 5% in 1962 to 44.1% in 2014 (FAO 2016). While remembering the advantages of aquaculture, we must not forget the other side of this reality. The rapid growth of aquacultural productions raises several concerns related to the quality and safety of fish. Similar to other sectors of animal production, fish production also uses intensive and semi-intensive practices, leading to a higher concentration of animals in small spaces of water, substantially increasing the risk of contagious diseases (EFSA Journal 2017).

In fact, disease is one of the major disadvantages of aquaculture; fish species reared under crowded and stressful conditions are more susceptible to bacterial infection, causing major stock losses, and the prophylactic and therapeutic use of antimicrobials and chemical disinfectants is currently employed to control disease outbreaks (Cabello *et al.*, 2013).

1.4 CURRENT TREATMENT METHOD IN AQUACULTURE INDUSTRY

In the aquaculture industry, chemicals and drugs are consistently used due to their efficiency in promoting productivity, aquatic growth, and disease control. They can be found in the form of antibiotics, pesticides, antifoulants (Bridson *et al.*, 2020; Quinones *et al.*, 2019), anaesthetics (Aydin and Barbas, 2020; Purbosari *et al.*, 2019), and disinfectants (Stockton-Fiti and Moffitt, 2017; Yun *et al.*, 2020). Many studies have reported the detection of antibiotic residues in samples from aquaculture sediment (Kumar *et al.*, 2019; Siedlewicz *et al.*, 2016). These substances are later discharged to external water bodies and eventually affect environmental and human health. China is the largest antibiotic producer and consumer, and antibiotic residues have been highlighted as a serious issue here (Han *et al.*, 2020; Liu *et al.*, 2017).

Disinfection is commonly used as a disease management tool in aquaculture and is designed to prevent, control, and eradicate diseases. This basic principle involves safe concentrations to treat and neutralise pathogens and to thereby avoid polluting the environment, which can endanger water bodies and humans (Liu *et al.*, 2016; Muniesa *et al.*, 2019). Some disinfectant formulations contain endocrine compounds that may affect not only fish but other aquatic microorganisms as well (Kar *et al.*, 2020; Yun *et al.*, 2020). Vaccination has also been regarded as a potential treatment against diseases outbreaks in aquaculture. However, commercial vaccines are too expensive for a widespread use by fish producers and they have the downside that a single vaccine is effective against only one type of pathogens (Sakai, 1999; Pasnik *et al.*, 2005; Harikrishnan *et al.*, 2011a). Finally, there is an increasing interest in consuming organic and environmentally friendly food. Thereafter, the limitation of chemical products in aquaculture and the use of natural treatments could enhance the consumption of aquaculture products.

1.5 POTENTIAL OF PLANT EXTRACTS IN AQUACULTURE

Plant extracts have been reported to favor various activities like anti-stress, growth promotion, appetite stimulation, enhancement of tonicity and immunostimulation, maturation of culture species, aphrodisiac and anti pathogen properties in fish and shrimps aquaculture due to the active principles such as alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids or essential oils (Citarasu, 2010; Chakraborty and Hancz, 2011). Besides, their use could reduce costs of treatment and be more environmentally friendly as they tend to be more biodegradable than synthetic molecules and they are less likely to produce drug resistance on parasites due to the high diversity of plant extract molecules (Logambal *et al*, 2000; Blumenthal *et al.*, 2000; Olusola *et al.*, 2013).

1.5.1 Plant extract as immunostimulants

The immune system is classified into innate (non-specific) and adaptive (specific) immune systems. The innate immune system is the first line of defense against invading pathogens and their major components are macrophages, monocytes, granulocytes, and humoral elements, including lysozyme or complement system (Harikrishnan *et al.*, 2009b). An immunostimulant is a substance that enhances the defense mechanisms or immune response (both specific and non-specific), thus rendering the animal more resistant to diseases and external aggressions (Anderson, 1992). An increasing interest in the use of plant extracts as fish immunostimulants has arisen in the last decade (Galina *et al.*, 2009 ; Vaseeharan and Thaya, 2013). Several studies have monitored the immunological parameters after intraperitoneal injection or orally administered plant extracts on distinct fish species and they found that treated fish showed increased lysozyme activity, phagocytic activity, complement activity, increased respiratory burst activity and increased plasma protein (globulin and albumin) (e.g. Dügenci *et al.*, 2003; Yuan *et al.*, 2007; Wu *et al.*, 2010). Lysozyme plays an important role in the defense of fish by inducing antibacterial activity in the presence of complement (Harikrishnan *et al.*, 2012b). Phagocytosis is

one of the main mediators of innate immunity to pathogens, while respiratory burst is also a crucial effector mechanism for limiting the growth of fish pathogens (Divyagnaneswari *et al.*, 2007). Increase in plasma protein, albumin and globulin, is considered as a strong innate response in fishes.

1.6 FRUIT PROCESSING BY-PRODUCTS

Fruits undergo various industrial processing, giving various waste materials with different forms and shapes. Fruits by-products have been reported as potent sources of bioactive compounds such as phenolic compounds, minerals, fatty acids, volatiles, anthocyanins, vitamins, etc. (Galali *et al.*, 2020, Rico *et al.*, 2020). Compared with the edible parts of the fruits, the non-edible parts as peels or skin portions, often comprise higher amounts of bioactive constituents (Gorinstein *et al.*, 2001). For instance, former studies found that peels of apple, grapes, citrus fruits, and the seeds of jackfruit, avocado, and mango have a higher amount (10–20%) of polyphenolic compounds than pulp (Ben-Othman *et al.*, 2020).

Former authors reported that the red apple peel contains bioactive contents such as triterpenoids, flavonoids, organic acids, and plant sterols, whereas the majority of polyphenols are concentrated in the peel of apple fruits (Qiang *et al.*, 2019). Further, the peels of sweet orange contain main bioactive components such as pectin, phenolic, alkaloids, tannins, and flavonoids (Hosseini *et al.*, 2020). Pomegranate peel is also a good source of flavonoids, phenolic acids, and tannins (ellagitannins such as punicalin, punicalagin, gallic acid, and ellagic acid) (Monir *et al.*, 2020). Moreover, essential oils produced from fruit peel are a rich source of antioxidants and immunomodulators. Bergamot essential oil contains many volatile components such as limonene, linalyl acetate, linalool, γ -terpinene, and β -pinene (Kesbiç *et al.*, 2020). Fruit pomace is the most common by-product that represents about 20–25% of the fresh fruit weight and results from juice extraction from fruits. It is a mix of pulp, skin, seeds, and stem with a large amount of water and fermentable sugars (Colovi *et al.*, 2019). Numerous reports stated the potential of fruit pomace as a food ingredient in the ration of dairy cattle (Ghoreishi *et al.*, 2007),

pigs(Rao *et al.*, 2003), rabbits (Fanimio *et al.*, 1996), and poultry (Wallace *et al.*, 2010).

1.6.1 Mode of action

The fruits by-products are enriched with various bioactive components that possess potent biological values. These compounds are recognized for their antioxidants (Eid *et al.*, 2021, Souza *et al.*, 2019), anti-inflammatory (Harikrishnan *et al.*, 2021), antibacterial (Harikrishnan *et al.*, 2021, Giriet *et al.*, 2016), as well as stimulation of immune response (Rattanaichai *et al.*, 2014) and modulation of gut microflora (Meng *et al.*, 2019). In this sense, the underlying mechanisms that explain their different actions on the aquatic organisms will be addressed to understand their role in improving aquaculture nutrition and health.

1.6.2 Pomegranate peel

Pomegranate (*Punica granatum* L.) constitutes a naturally rich source of phenolic compounds. It is usually consumed fresh or commercialized in the form of juice. The global production of pomegranate was estimated to be around ~3.8 million tons in 2017 (Kahramanoglu, 2019). The pomegranate juice processing generates large amounts of wastes composed mainly of peel (~78%), which is a potential material for further valorization (Kaderides *et al.*, 2020). However, most of these wastes are generally used as animal feed or discarded into landfill sites (Cam *et al.*, 2014). Pomegranate peels contain high amounts of secondary metabolites, mainly phenolic acids (e.g. gallic acid, ellagic acid, caffeic acid), flavonoids [i.e. flavonols (e.g. catechin, galocatechin, epicatechin), anthocyanins) and hydrolysable tannins [i.e. ellagitannins (e.g. punicalagin), gallotannins] (Kaderides *et al.*, 2020). Numerous in vitro and in vivo studies have associated the presence of these phenolic compounds with a wide range of biological activities and health benefits, such as antioxidant, anti-inflammatory, antimutagenic, anticarcinogenic, antihypertensive. Moreover, their presence has been associated with the prevention and treatment of several chronic pathological conditions including cardiovascular diseases, diabetes, obesity and Alzheimer's (Kandylis & Kokkinomagoulos, 2020; Ko, Dadmohammadi, &

Abbaspourrad, 2021). In this view, pomegranate peel bioactive compounds could be exploited as functional ingredients and offer additional value to the pomegranate industry.

CHAPTER 2

LITERATURE SURVEY

Sanjay kumargupta *et al.*, 2023 reported study, Pomegranate (*Punica granatum*) peel extract represented remarkable favourable variations in growth, haemato-immunological parameters and cytokine gene expression of *L. rohita* fingerlings. Thus, the dietary supplementation of PPE at 0.5% could effectively enhance different aspects of innate immunity alongwith disease resistance to *A. hydrophila* infection in *L. rohita* fingerlings.

Walid Monir *et al.*, 2020 reveled that fish diet supplementation with pomegranate PE enhances immunity and increases the protection of *O. niloticus* against *A. hydrophila*. Fish diet supplementation with Moringa LE not only enhanced the immunity and increased the protection of *O. niloticus*, but also improve the growth performance of Nile tilapia (*O. niloticus*) fishes. Supplementation of Moringa LE at 0.25% kg⁻¹ of feed diet could enhance the challenge of fish against infection with the pathogenic bacteria, and achieved mortality rate at 10% as compared to 80% obtained without Moringa LE supplementation to fish diets. It could be recommended that, adding Moringa LE to fish diet exhibits the significant improvement of growth parameters, enhancement of the immunity of fish against bacteria, reduction of the cost, and maximizing profits.

Shuai Zhuang *et al.*, 2019 reported of the evaluation, PPE can be a potential preservative for chillstored bighead carp fillets, although its unfavorable color needs to be modified and improved. In addition, further studies are recommended to

elucidate the preservative effects of specific components in PPE and, especially, the mechanisms that inhibit spoilage bacteria like *Acinetobacter* and *Shewanella*.

Elisabete M.C. Alexandre *et al.*, 2018 The reported study aimed to assess the effect of high pressure (300 and 600 MPa) and enzymatic extraction (pectinase and cellulase) on the phenolic compounds profile, antioxidant capacity and antimicrobial activity of extracts from pomegranate by-products. Antimicrobial activity against eight different strains of pathogenic and contaminant bacteria and against five beneficial bacteria including *Lactobacillus* and *Bifidobacterium* strains were determined.

Adem Yavuz sönmez *et al.*, 2022 The reported study of Both *Veratrum album* and pomegranate peel extract supplementation at varying doses increased the overall immune status and antioxidant activities of rainbow trout fingerlings in a short period. Although all results were obtained under laboratory conditions, it may be suggested that short-term usage of feed supplemented especially with pomegranate peel extract could be used to improve immune responses. However, its long-term usage is not suggested. High doses of veratrum are not suggested as an additive at all. Pomegranate peel, when supplemented at 500 mg/ kg, in particular, can be beneficial as it lowers the FCR and improves the overall feed efficiency. Long-term effects are yet to be determined for different fish and feed combinations as these by-products of the food industry have huge potentials to be included in the fish feed as additives. Further studies may investigate the effects of these plant extracts on disease resistance.

Heba S. Hamed *et al.*, 2021 The reported study examined the protective role of PP powder to alleviate the toxic effects of AgNPs in Nile tilapia. The obtained results showed that dietary PP powder did not improve fish growth but effectively stimulated the oxidant/antioxidant and immuno-biochemical biomarkers of Nile tilapia. AgNPs exposure significantly suppressed fish growth, antioxidants, and immunity. The PP-

enriched diet mitigated the negative impacts of AgNPs toxicity and normalized fish immunity to levels similar to the control group.

Enayat Berizi *et al.*, 2017 The reported study showed the best performance to prevent the oxidation of fats and protein was in the chitosan combined with 4% PPE, however, due to its undesirable color, the chitosan combined with 2% PPE was preferred. A considerable improvement in the chemical, microbial, sensory and textural properties was shown in the Ch + PPE2 groups. Therefore, this active coverage was suggested as safe to store rainbow trout up to 6 months frozen storage.

Hamidreza Ahmadniaye Motlagh *et al.*, 2020 The reported study showed that supplementation of *C. auratus* diets by lower than 4% would not adversely affect growth. Furthermore, gut microbial analyses indicated that enteric gram-negative bacteria and total fungi count decreased and increased, respectively, under the influence of PPE (2 and 4%). Thus, dietary administration with 2% of PPE will modify gut bacteria without any adverse effect on fish growth. It appears that the supplementation of aquatic feed with this extract may be effective in producing functional diets. Finally, there is a suggestion to evaluate the other effects of PPE on the health of other tissues such as intestine and liver.

Ahmed Ali Badrey *et al.*, 2019 The study reported that the effects of dietary supplementation with the natural additive PP on haematology, blood biochemistry and immune status in monosex Nile tilapia (*Oreochromis niloticus*). Cholesterol levels were reduced by PP, while triglyceride levels were significantly increased. In addition, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were significantly reduced in the blood of monosex Nile tilapia fed diets with different concentrations of PP. Immunological parameters (IgM levels and lysozyme activity) were significantly improved by PP supplementation. These results indicate that

addition of PP to monosex *O. niloticus* diets improved immune status and liver and kidney function without any adverse effects on health status.

Mohamed M. Toutou *et al.*, 2019 The study reported that the fish fed on 5% and 3% PP levels exhibited the best performance and the best feed utilization indices. This was evidenced by the best protein efficiency ratio PER and the highest energy utilization for those fish. This was supported also by the increase in the length of villi and the number of goblet cells of monosex *O. niloticus* fed on 3 or/and 5% level PP. It is recommended to add the PP to the diets of monosex *O. niloticus* up to 5% to enhance body composition of fish, feed utilization and fish integrity with increasing economic efficiency.

Ebtehal E.Hussein *et al.*, 2023 The study reported that diet supplemented with pomegranate peel (3, 6, and 9 g/kg diet) as identified as POM3, POM6 and POM9 were fed to Nile Tilapia fries for 84 days to evaluate the nutritional values, antioxidant activity, histology of liver and intestine and growth of mono-sex Nile tilapia (*Oreochromis niloticus*) fries. No significant changes ($P \geq 0.05$) in the final weight, weight gain, and specific growth rate (SGR) were seen in all treatments. Meanwhile, a decreasing trend was detected in feed intake by increasing the pomegranate peel inclusion. Diet supplemented with pomegranate peel (3, 6, and 9 g/kg diet) as identified as POM3, POM6 and POM9 were fed to Nile Tilapia fries for 84 days to evaluate the nutritional values, antioxidant activity, histology of liver and intestine and growth of mono-sex Nile tilapia (*Oreochromis niloticus*) fries. No significant changes ($P \geq 0.05$) in the final weight, weight gain, and specific growth rate (SGR) were seen in all treatments. Meanwhile, a decreasing trend was detected in feed intake by increasing the pomegranate peel inclusion.

Mahwish Safdar *et al.*, 2021 The study reported that peel extract of both pomegranate varieties exhibited maximum antioxidant and antimicrobial activity and

total phenolic contents. The results also showed that using GC–MS and IR spectroscopy was an extremely effective method for determining the presence or absence of a wide variety of phytochemicals and functional groups in both varieties. Hence, this study also helps the researchers and industry to recognize the importance of pomegranate peel extracts and their health benefits used as antacids and laxatives in the pharmaceutical industry.

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To extract and characterize the bioactive compounds from pomegranate peel and analysing the disease resistance activity for aquaculture application

3.2 OBJECTIVE

- ❖ Collection of pomegranate peel from natural environments
- ❖ Extraction of immunostimulants products using various organic solvents
- ❖ Screening of antimicrobial activity against fish pathogen
- ❖ Characterization of active products by FTIR, and GC-MS.
- ❖ Evaluation of toxicity assay and disease resistance activity using fish model

CHAPTER 4

MATERIALS AND METHODS

4.1 SAMPLE COLLECTION

Pomegranate (*Punica granatum L.*) fruit were collected from Kolathur surrounding area of Chennai, Tamil nadu, India. The fruits were washed with distilled water and peeled, and their edible portions was carefully separated (Fig 1). the peels are manually air-dried under the shade for 2 weeks and the dried pomegranate peels crushed, powdered, sieved and stored at room temperature.



Fig 4.1 Pomegranate peel air –dried

4.2 EXTRACTION OF BIOACTIVE COMPOUNDS

The extraction was carried out as follows: grounded pomegranate peels 20g were mixed with different solvent such as ethanol, methanol, acetone, water and mixture of ethanol and water (3:7, 7:3 and 5:5) and kept it in shaking incubator for 24 hours (Fig 2). After incubation, different solvent extract were filtered through Whatman No.1 filter paper and transferred into petri dish for air dry, the extract were collected in glass vials. The high amount of extract were collected from ethanol extract compared to the rest of the extract.



Fig 4.2 Extraction of bioactive compound from pomegranate peel

4.3 CHARACTERIZATION OF BIOACTIVE COMPOUND

4.3.1 GC-MS analysis

The bioactive compounds was extracted from the pomegranate peel by using ethanol solvent and characterized by Gas Chromatography-Mass Spectrometry (GC-MS) analysis to identify the active compounds. The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. The 1µL of extract sample injected into the instrument the oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min⁻¹; and 300 °C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 230 °C; ion source temperature 230 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

4.3.2 FTIR analysis

The ethanol extracted bioactive compound from the pomegranate peel was analyzed qualitatively for the active compounds by Fourier Transform Infra-Red (FT-IR) method. Two milligram of dried fish fecal sample was mixed with 100 mg KBr and compressed in the form of KBr pellet The infrared (IR) spectrum was recorded in the wave length ranging between 4000 and 400 cm⁻¹ using FTIR spectrometer (Bruker spectrophotometer) (Amir *et al.*, 2005 ; Gong *et al.*, 2020).

4.4ANTIMICROBIAL SUSCEPTIBILITY TEST

4.4.1 Isolation of microorganisms

Four aquatic fish pathogens (*Aeromonas hydrophila*, *Aeromonas caviae*, *Staphylococcus aureus*, *Edwardsiella tarda*) were collected from Centre for Aquaculture, Sathyabama Institute of Science and Technology, Chennai. The subculture plates were prepared by inoculating the mother culture of four bacterial pathogens in the nutrient agar plates. Then the cultured plates were incubated at 37°C for 24 hours.

4.4.2 Preparation of nutrient broth

Suspend 1.3 g of nutrient broth in 100 ml of distilled water and sterilized by autoclaving at 121°C for 15 minutes. A loop of culture is inoculated in 10ml of nutrient broth in a test tube and incubated at 37°C for 24 hours.

4.4.3 Agar well diffusion method

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts. Similarly to the procedure used in disk-diffusion method, the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (20–100 µL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested.

4.4.4 Preparation of Mueller hinton agar

Suspend 3.9g of Mueller Hinton Agar in 100ml of distilled water and sterilized by autoclaving at 121°C for 15 minutes. Pour the liquid in petri dishes and leave for solidifying.

The standardized suspension was used to inoculate the microorganisms on the surfaces of Mueller Hinton agar plates using sterile cotton swab. The plates were left for 20 minutes; using a 100 µL pipette tip make a hole with a diameter of 6mm and extract solution at desired concentration (10µL, 20µL and 50µL) is introduced into the well. Then the plates were incubated at 37°C for 24 hrs and then were examined for the zone of inhibition around the well.

4.5 EXPERIMENTAL FISH

A total of 100 apparently healthy live koi carp (*Cyprinus carpio*, L) with an average body weight of 4.4 ± 0.5 g were obtained from a private fish farm in Kolatur, Chennai, India. The fish were kept in a recirculation system. High water quality was ensured, and the system was thermostatically controlled at $25 \pm 1^\circ\text{C}$. The fish were divided into 3 groups, each of which was divided into subgroups. Each subgroup had three replicates (10 fish/replicate). The fish were allowed to acclimate to the laboratory conditions for three weeks before the start of the experiment. The experiment carried out at form Centre for Aquaculture, Sathyabama Institute of Science and Technology, Chennai.

4.6 FEED PREPARATION

The experimental feeds were prepared with basic ingredients such as wheat flour, rice bran, tapioca flour, ragi flour, fish meal and groundnut oilcake (Table 1 and Fig. 1). They were split into three equal portions. These ingredients were mixed until very soft biscuit-textured dough was obtained. The material was then steam sterilized (autoclaved at 120 °C for 20 min) and pelletized by hand to get pellets of 3 mm size. The pellets were dehydrated in a forced-air oven at 55–60 °C for 48 h when the moisture content <15%. After drying, the basal diet supplemented with pomogranate

peel extract at rates of 0.1, 0.3 and 0.5% (G1, G2 & G3) respectively. After that, dough was heated in a drying oven at 35 °C for 30 min and asked by crumbling and then stored at room temperature.



Fig 4.3 Feed preparation according to the concentration

Table 4.1: Shows Formulation of Experimental Diets (g/100g)

Control	Group1 (G1)	Group2 (G2)	Group3 (G3)
Soybean (15.22)	Soybean (15.22)	Soybean (15.22)	Soybean (15.22)
Fishmeal (15.22)	Fishmeal (15.22)	Fishmeal (15.22)	Fishmeal (15.22)
Groundnut oilcake(11.28)	Groundnut oilcake(11.28)	Groundnut oilcake(11.28)	Groundnut oilcake(11.28)

Wheat flour (13.04)	Wheat flour (13.04)	Wheat flour (13.04)	Wheat flour (13.04)
Tapioca flour (13.04)	Tapioca flour (13.04)	Tapioca flour (13.04)	Tapioca flour (13.04)
Rice bran (12.2)	Rice bran (12.2)	Rice bran (12.2)	Rice bran (12.2)
Vitamins (5. 0)	Vitamins (5. 0)	Vitamins (5. 0)	Vitamins (5. 0)
Nil	PPE (0.1%)	PPE (0.3%)	PPE (0.5%)

4.7 GROWTH PERFORMANCE AND CONDITION FACTOR OF KOI CARP FISH

For each experimental treatment, 10 fish were randomly selected bi-weekly to estimate the periodical gain in weight and length. At the end of 30 days experimental period, koi carp fish were anesthetized by clove oil (Himedia, Mumbai, India, 50 $\mu\text{L L}^{-1}$), collected, counted, and weighed. The growth performance and feed utilization parameters were determined as follows:

- Weight gain (g fish^{-1}), $\text{WG} = \text{Wt} - \text{W0}$;
- Average daily gain $\text{ADG g fish}^{-1} \text{ day}^{-1} = \text{Wt} - \text{W0 days}^{-1}$;
- Specific growth rate ($\% \text{ day}^{-1}$);
- $\text{SGR} = 100 \times \ln \text{Wt} - \ln \text{W0} / \text{experimental period}$, where: W0: initial fish weight (g), Wt: final fish weight (g), $\ln$: natural logarithm;
- Survival rate (%) = $100 \times (\text{final number of fish} / \text{initial number of fish})$.
- Length gain (cm fish^{-1})= $\text{Lt} - \text{L0}$; condition factor (K) = $100 \times \text{W1} / \text{Lt}^3$ where, Lt: final fish length (cm), L0: initial fish length (cm).

4.8 STATISTICAL ANALYSIS

Statistical analyses were carried out on various parameters that were measured on the plants to evaluate the effect of each treatment. Data was presented as mean value $\pm$ standard deviation (error bars). Analysis of variance (ANOVA) was used and

Tukey's honest significant difference ($P < .05$) multiple range test was conducted to determine differences between treatments.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 EXTRACTIVE VALUES OF DIFFERENT SOLVENT BASED EXTRACTS OF POMEGRANATE PEEL

The extractive values of different parts of pomegranate peel using different solvents such as aqueous, ethanol, acetone, methanol, and mixture of ethanol & water. The extractive value was higher in ethanol extract of pomegranate peel (18.2%) followed by methanol extract of pomegranate peel (14.9%), acetone extract of pomegranate peel (7.9%), mixture of ethanol and water (3:7) extract of pomegranate peel (6.9%), ethanol and water (5:5) extract of pomegranate peel (6.9%) and ethanol and water (7:3) extract of pomegranate peel (6.9%).

Showed that high polarity solvents can resolve variety of plant phytochemicals than the other solvents. Found that the extractive value was found to be high in polar solvents when compared to the other solvents. Found that aqueous extract yielded highest extraction. This result was similar with also found that increasing polarity of the selected solvent increased the extractive values.

5.2 GC-MS ANALYSIS

The GC-MS study revealed that the presence of chemical compounds in the pomegranate peel extract was identified with the help of the NIST library (Table 6) (Fig. 3). The peak at the retention times of 37.35, 37.58, 44.206, were confirmed as 9-Octadecenoic acid, cis-9-Hexadecenal, and 92 E, E, Z-1, 3, 12- Nonadecatriene-5, 14-diol. The compounds may act as highly responsible agent for their antimicrobial and antioxidant activity.

Table 5.1 Major components from the GC-MS analysis of the ethanol extract of pomegranate peel.

Peak	R.Time	Area	Area%	Name
1	37.35	50556486	3.62	9-Octadecenoic acid
2	37.58	47779393	3.42	cis-9-Hexadecenal
3	37.68	82103601	5.88	Oleic Acid
4	37.78	63337840	4.53	Oleic Acid
5	37.845	75279777	5.39	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-
6	38.028	95440419	6.83	9-Octadecen-1-ol, (Z)-
7	38.137	56673200	4.06	9-Octadecen-1-ol, (Z)-
8	38.197	78557038	5.62	14-METHYL-8-HEXADECYN-1-OL #
9	38.255	41528043	2.97	Oxacyclohexadecan-2-One
10	38.292	65256175	4.67	Oleic Acid
11	38.35	14012941	4.1	03 9-Octadecenoic acid, (E)-
12	44.089	76260698	5.46	Ethyl (9z,12z)-9,12-Octadecadienoate #
13	44.206	33421400	7.23	92 E,E,Z-1,3,12-Nonadecatriene-5,14-diol
14	44.295	15049134	6.1	77 9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-
15	44.653	39815289	2.85	Glycidol stearate

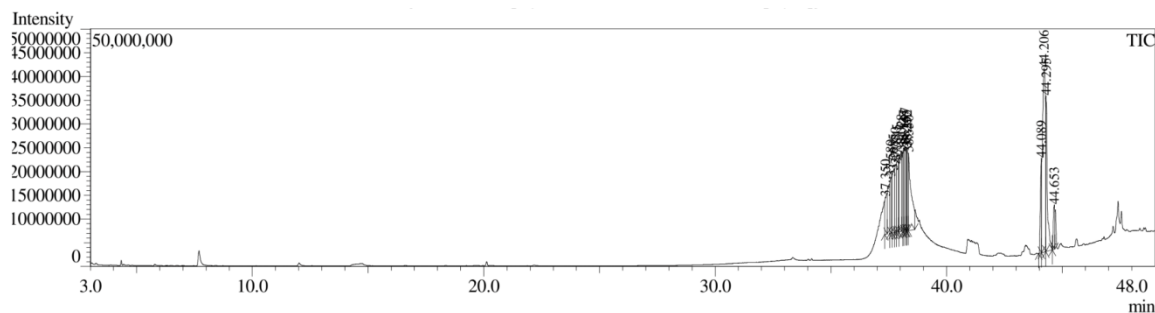


Fig 5.1 Structural characterization of pomegranate peel active fraction by GC-MS analysis.

5.3 FTIR ANALYSIS

The IR spectrum of the ethanol extract of pomegranate peelsample showed in Table 5 and Fig. 1. The main absorption band at around 3329 cm^{-1} , which is strong, broadband and attributed to N-H stretching showed the presence of aliphatic primary amine. The absorption band at 2972.73 cm^{-1} was identified as O-H stretching and showed the presence of conjugated alcohol, the band at 1721.16 cm^{-1} was recognised as C=C bending exhibiting the presence of alkene and the band at 877.45 cm^{-1} was recognised as C-Cl bending exhibiting the presence of Alkyl Halide.

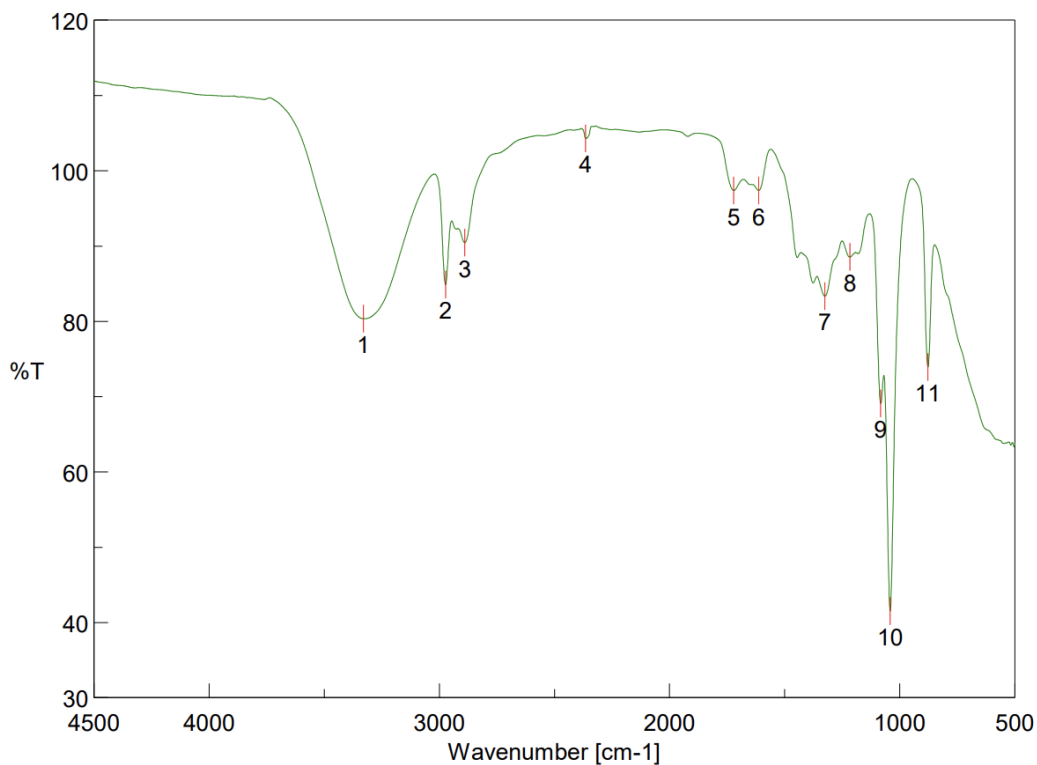


Fig 5.2 Structural characterization of pomegranate peel active fraction by FTIR analysis.

Table 5.2 IR absorption frequencies of organic functional groups of pomegranate peel extract using FTIR

No. Position	Intensity No. Position	Intensity	Reported	Functional group	
1	3329.5	80.3198	3,300–3,500	N–H	Amine
2	2972.73	84.8519	2,500–3,300	O–H	Alcohol
3	2889.81	90.4297	2,500–3,300	O–H	Alcohol
4	2364.3	104.279	2,500–3,300	O–H	Alcohol
5	1721.16	97.3596	1,400–1,600	C=C	Alkene
6	1612.2	97.3509	1,400–1,600	C=C	Alkene
7	1325.82	83.3124	1,000–1,300	C–O	Alcohol
8	1216.86	88.5095	1,000–1,300	C–O	Alcohol
9	1082.83	69.0549	1,000–1,300	C–O	Alcohol
10	1041.37	41.4864	1,000–1,300	C–O	Alcohol
11	877.452	73.8801	600–800	C–Cl	Alkyl Halide

5.4 ANTIMICROBIAL SUSCEPTIBILITY TEST

The pomegranate peel extract exhibited most significant activity against aquatic fish bacterial pathogens. The extract showed maximum activity against the tested pathogens. The significant zone of inhibition was observed against the bacterial pathogens *Aeromonas caviae* (12.13 mm), *Staphylococcus aureus* (12.77 mm), *Edwardsiella tarda* (13.96 mm) and *Aeromonas hydrophila* (13.6 mm) (Fig 5).

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration of the ethanol extract of pomegranate peel against *Aeromonas caviae*, *Staphylococcus aureus*, *Edwardsiella tarda* and *Aeromonas hydrophila*. The ethanol extraction helped to control *Aeromonas caviae*, *Staphylococcus aureus*, *Edwardsiella tarda* and *Aeromonas hydrophila* at the minimal level of 10µg due to the presence of antimicrobial active principles (Table 4).

Table 5.3 In vitro antagonistic screening of pomegranate peel extract against bacterial pathogens

Bacterial Pathogens	10 µg	20 µg	50 µg
<i>Aeromonas hydrophila</i>	8.73±0.64	10.8 ± 1.76	13.6±1.25
<i>Aeromonas caviae</i>	10.0 ± 0.54	10.67 ± 1.50	12.13±1.03
<i>Edwardsiella tarda</i>	9.0±1.25	10.57 ± 1.55	13.96±1.54
<i>Staphylococcus aureus</i>	10.30±1.30	11.23 ± 1.11	12.77±1.46



(a)



(b)



(c)



(d)

Fig 5.3 Antimicrobial activity of bioactive compound by agar well diffusion method (a) *Aeromonas caviae* (b) *Aeromonas hydrophila* (c) *Edwardsiella tarda* (d) *Staphylococcus aureus*

5.5 GROWTH PERFORMANCE

Results of growth performance are illustrated in Table 5. There were significant ($P < 0.05$) differences among treatments in terms of final weight, weight gain, average daily gain (ADG), specific growth rate (SGR), final length, length gain and condition factor (K). Fish fed with experimental diet showed the highest values of growth performance, and lowest values of condition factor.

Adult fish can grow on vegetable diets, however juvenile fish require a greater protein content in their feeds for better growth. Furthermore, improved growth performance may be attributed to feeding fish live natural food rather than commercial diets which are dependent on the palatability, quality, and adequate quantity of feed, size, type, and physical appearance of the feed, as well as other factors such as attractiveness, feed acceptability, and assimilation of nutrients.

Table 5.4 Growth performance, Survival rate and feed utilization of *C. carpio* fed with experimental diets for 30 days

Parameters	Control	Group 1	Group 2	Group 3
Initial weight (g)	4.26±0.44	4.4±0.75 ^a	4.32±0.83 ^b	4.4±0.75
Final weight (g)	6.4±0.06	8.5±0.65 ^a	9.1±0.24 ^b	7.12±0.42 ^a
Weight gain	50.23±0.23	93.18±0.13 ^a	110.64±0.31 ^b	61.81±0.53 ^a
Specific growth rate	7.13±0.34	6.83±0.33 ^a	7.96±0.13 ^b	9.06±0.28 ^a
FCR	0.07±0.01	0.08±0.40 ^a	0.06±0.15 ^b	0.06±0.02 ^a
Survival	80	90	90	80

Values are represented as mean ± SD (n = 3), Significant difference ($p < 0.05$) vs control

CHAPTER 6

SUMMARY AND CONCLUSION

The bioactive compound extractive value was higher in ethanol solvent extract of pomegranate peel (18.2%) followed by methanol, acetone, mixture of ethanol and water (3:7), (5:5), (7:3) extract of pomegranate peel. The GC-MS study revealed that the presence of chemical compounds in the pomegranate peel extract was identified as 9-Octadecenoic acid, cis-9-Hexadecenal, and 92 E, E, Z-1,3,12-Nonadecatriene-5,14-diol. The compounds may act as highly responsible agent for their antimicrobial and antioxidant activity. The FTIR spectrum of the ethanol extract of pomegranate peel sample showed the presence of aliphatic primary amine, conjugated alcohol, alkene and Alkyl Halide. The pomegranate peel extract exhibited most significant activity against aquatic fish bacterial pathogens such as *Aeromonas caviae*, *Staphylococcus aureus*, *Edwardsiella tarda*, and *Aeromonas hydrophila*. The extract showed maximum activity against the *Edwardsiella tarda* pathogens. Experimental fish diet prepared supplemented with pomegranate peel extract at rates of 0.1, 0.3 and 0.5% (G1, G2 & G3) respectively. Fish fed with experimental diet showed the highest values of growth performance, and lowest values of condition factor. In conclusion, this study declared that the addition of pomegranate peel extract

(PPE) in *Cyprinum carpio* diets adverse effects on growth parameters or health status. In addition, it contains bioactive compounds responsible for antimicrobial and antioxidant activity that improved immune status. PPE has been suggested as immunostimulant due to their biological effects.

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