Bindura University of Science Education

Faculty of Science

Biological Sciences Department

Investigating the efficacy of *Punica Granatum* (pomegranate) peel extract against *Pseudomonas aeruginosa,* a causative agent of airsucculitis in poultry

By

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A research project submitted in partial fulfillment of the requirements for the Bachelor of Science Honours Degree in Biotechnology.

Approval form

The undersigned certify that they have read the dissertation titled '**Investigating the efficacy of *Punica Granatum* (pomegranate) peel extract against *Pseudomonas aeruginosa,* a causative agent of airsucculitis in poultry'.** and confirm that it is suitable for submission to the Biological Sciences Department, Faculty of Science and Engineering, for assessment.

Signature of student: Date: 16/06/2026



Signature of Supervisor:  Date: 20 June 2026

Signature of Chairperson of Department:  Date: 13 August 2026

Declaration

I, Tadiwanashe Elton Mhashu (B224374B) declare that this research herein is my own work and has not been plagiarized from another source(s) without acknowledgement of the concerned author(s) either electronically or otherwise

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I, Dr. P. Jinga, declare that I have supervised this thesis and I am satisfied that it can be submitted to the Biological Sciences Department, Faculty of Science and Engineering, at Bindura University of Science Education.

Signature:  Date: 20 June 2026

Dedication

I dedicate this project to my mother, Mary Mhashu for being there for me when I needed her the most

Acknowledgments

Firstly, I would like to the God for being an unwavering pillar of spiritual strength throughout my project. I would also like to thank my parents for their financial and emotional support, my supervisor Dr P. Jinga for his unwavering mentorship and guidance, and Bindura University for providing me with the resources required for this project.

List of abbreviations

AAAP – American Association of Avian Pathologists

AMR – Antimicrobial Resistance

CFU – Colony Forming Unit

CLSI – Clinical and Laboratory Standards Institute

FAO – Food and Agriculture Organization of the United Nations

MBC – Minimum Bactericidal Concentration

MHA – Mueller-Hinton Agar

MIC – Minimum Inhibitory Concentration

NA – Nutrient Agar

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Abstract

The increasing prevalence of antimicrobial resistance among bacterial pathogens has created a need for the exploration of alternative antimicrobial agents from natural sources. *Pseudomonas aeruginosa* is an opportunistic pathogen associated with numerous healthcare-associated infections and is known for its resistance to multiple antimicrobial agents. This study evaluated the antimicrobial activity of ethanolic *Punica granatum* (pomegranate) peel extract against *P. aeruginosa* and investigated the presence of selected phytochemical compounds responsible for its antibacterial activity.

Fresh pomegranate peels were collected, air-dried, powdered, and extracted using 50% ethanol through cold maceration. Qualitative phytochemical screening was conducted to detect flavonoids, phenols, and tannins. Antibacterial activity was assessed using the agar well diffusion method against a standardized 0.5 McFarland suspension of *P. aeruginosa*. Extract concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL were tested alongside ciprofloxacin as a positive control and sterile distilled water as a negative control. The minimum inhibitory concentration (MIC) was determined using a broth microdilution assay. Phytochemical screening confirmed the presence of flavonoids, phenolic compounds, and tannins in the extract. The extract demonstrated concentration-dependent antibacterial activity, with mean zones of inhibition ranging from 2.63 ± 0.45 mm at 6.25 mg/mL to 15.1 ± 0.2 mm at 100 mg/mL. Ciprofloxacin produced the largest inhibition zone (24.07 ± 0.25 mm), while the negative control produced no inhibition. Statistical analysis revealed significant differences among treatment groups ($p < 0.001$). The MIC of the extract against *P. aeruginosa* was determined to be 25 mg/mL. The findings indicate that ethanolic *P. granatum* peel extract possesses measurable antibacterial activity against *P. aeruginosa*, likely due to the presence of flavonoids, phenols, and tannins. The study supports the potential use of pomegranate peel as a source of natural antimicrobial compounds and provides a basis for further research into its therapeutic applications.

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Chapter 1

Introduction

1.1 Background

Poultry production in Zimbabwe plays a big role in food security and income generation for many households and industries (Gororo & Kashangura, 2016). However, it is continually challenged by infectious diseases that reduce productivity and increase economic losses (Zhou, Shen and Bi, 2020) Among these diseases, respiratory infections such as airsacculitis are of particular concern due to their impact on bird health, growth performance, and carcass quality (Glisson, 1998; Barnes et al., 2008).

Airsacculitis is a respiratory illness that is commonly associated with some bacterial pathogens, as well as in some cases, *P. aeruginosa*, which is an opportunistic microorganism widely distributed in the environment (Debnath, S. K et al.,2022). *Pseudomonas aeruginosa* is capable of causing severe infections in poultry, particularly under conditions of stress, poor hygiene, and compromised immunity (Gellatly & Hancock, 2013). Its ability to form biofilms and produce virulence factors contributes to persistent infections and complicates treatment strategies (Gellatly & Hancock, 2013).

The control of bacterial infections in poultry has traditionally relied on the use of antibiotics. However, the increasing emergence of antimicrobial resistance has reduced the effectiveness of these drugs, posing a major challenge to both animal health and public safety (Van Boeckel et al., 2015). The presence of antibiotic-resistant bacteria in poultry systems also raises concerns about the potential transmission of resistant strains to humans through the food chain (Marshall and Levy, 2011).

In response to these challenges, there has been growing interest in the use of plant-based antimicrobials as alternative therapeutic agents. Medicinal plants contain bioactive compounds such as tannins, flavonoids, and phenolics, which have demonstrated antimicrobial activity against a range of pathogens (Cowan, 1999; Daglia, 2012). These natural products are considered promising due to their availability, affordability, and reduced likelihood of inducing resistance.

One such plant is *Punica granatum* (pomegranate), whose peel has been reported to possess significant antimicrobial properties. The peel contains high concentrations of bioactive compounds, including punicalagin and ellagic acid, which contribute to its antibacterial activity against both Gram-positive and Gram-negative organisms (Al-Zoreky, 2009; Viuda-Martos et al., 2010). Despite these findings, limited studies have focused on its effectiveness against *P. aeruginosa* associated with airsacculitis in poultry.

Therefore, this study aims to investigate the efficacy of *P. granatum* peel extract against *P. aeruginosa*, with the goal of exploring alternative antimicrobial strategies for poultry disease management.

1.2 Aim of the study

The aim of this study is to investigate the efficacy of *P. granatum* peel extract against *P. aeruginosa*.

1.3 Problem statement

Airsacculitis is a significant respiratory disease affecting poultry and is associated with reduced productivity, increased mortality, and considerable economic losses within the poultry industry (Serbessa et al., 2023). The disease is frequently linked with opportunistic bacterial pathogens, including *P. aeruginosa*, which are widely distributed in soil, water, and farm environments. This bacterium can cause persistent infections in poultry, particularly when birds are exposed to

poor management conditions, environmental stress, or compromised immune systems (Lister et al., 2009; Serbessa et al., 2023).

The treatment of bacterial infections in poultry has traditionally relied on the use of antibiotics. However, the increasing emergence of antimicrobial resistance among bacterial pathogens such as *P. aeruginosa* has significantly reduced the effectiveness of many commonly used antibiotics (Breidenstein et al., 2011; Marshall & Levy, 2011). As a result, treatment failures, prolonged infections, and increased production costs have become more common in poultry production systems. Furthermore, the extensive use of antibiotics in animal agriculture raises concerns regarding the presence of drug residues in animal products and the potential transmission of antibiotic-resistant bacteria to humans through the food chain (Van Boeckel et al., 2015; Nhung et al., 2017).

Despite the growing need for alternative antimicrobial agents, relatively limited research has been conducted on the use of plant-derived extracts in controlling *P. aeruginosa* infections in poultry, especially in cases of airsacculitis. Although *P. granatum* peel extract has demonstrated antimicrobial activity against a variety of bacterial pathogens in previous studies (Al-Zoreky, 2009; Viuda-Martos et al., 2010), its efficacy against *P. aeruginosa* isolated from poultry has not been extensively investigated.

Therefore, there is a need to explore alternative, effective, and sustainable antimicrobial agents that may help manage bacterial infections in poultry. Investigating plant-derived antimicrobial compounds may contribute to reducing reliance on conventional antibiotics while improving disease control in poultry production. Consequently, this study aims to evaluate the antimicrobial efficacy of *P. granatum* peel extract against *P. aeruginosa* associated with airsacculitis.

1.4 Objectives

The specific objectives of this study are:

1.4.1 To prepare *P. granatum* peel extract.

1.4.2 To evaluate the antimicrobial activity of *P. granatum* peel extract against *P. aeruginosa.*

1.4.3 To determine the effectiveness of the extract at different concentrations.

1.5 Research questions

This study seeks to answer the following questions:

1. Was the *P. granatum* peel extract successfully obtained?

2. Does *P. granatum* peel extract exhibit antimicrobial properties against P. aeruginousa?

3. How does the effectiveness of the extract vary with concentration?

1.6 Hypothesis

P. granatum peel extract has no significant antimicrobial effect against *P. aeruginosa.*

1.7 Justification of the study

The poultry industry plays a large role in enhancing food security, improving nutrition, and generating income in Zimbabwe and other developing countries. Despite its importance, the productivity of this sector is significantly constrained by infectious diseases such as airsacculitis,

which contribute to substantial economic losses through increased mortality, decreased growth of the poultry, and carcass condemnation (Glisson et al., 2013; Food and Agriculture Organization, 2019). Addressing these disease-related challenges is essential for improving poultry health and ensuring sustainable production systems.

The growing prevalence of antimicrobial resistance (AMR) among bacterial pathogens, particularly *P. aeruginosa*, has diminished the efficacy of conventional antibiotics commonly used in poultry production (World Health Organization, 2023; Laxminarayan et al., 2013). This situation has created an urgent demand for alternative antimicrobial agents that are not only effective but also affordable and environmentally sustainable. Plant-based antimicrobials have emerged as promising candidates due to their rich composition of bioactive compounds and their relatively lower risk of inducing resistance compared to synthetic antibiotics (Cowan, 1999; Daglia, 2012).

Punica granatum peel, often discarded as agricultural waste, has been reported to contain potent antimicrobial phytochemicals, including tannins, flavonoids, and phenolic acids (Viuda-Martos et al., 2010). Investigating its efficacy against *P. aeruginosa* not only contributes to the development of alternative therapeutic strategies but also promotes the utilization of locally available natural resources. This approach may provide a cost-effective and sustainable solution for poultry farmers, particularly in resource-limited settings.

Furthermore, this study contributes to the expanding body of scientific knowledge on the antimicrobial properties of medicinal plants and supports global efforts to reduce dependence on synthetic antibiotics (World Health Organization, 2023). The findings may also have broader public health implications by helping to mitigate the transmission of antibiotic-resistant bacteria from poultry to humans (Marshall and Levy, 2011).

1.8 Limitations of the study

This study may be subject to several limitations. Firstly, the research is limited to in vitro analysis of the antimicrobial activity of *P. granatum* peel extract against *P. aeruginosa*. As a result, the findings may not fully reflect the effectiveness of the extract under in vivo conditions within poultry systems.

Another limitation is the potential variability in the composition of *P. granatum* peel due to differences in geographical origin, maturity stage of the fruit, and storage conditions. These factors may influence the concentration of bioactive compounds and, consequently, the antimicrobial activity of the extract.

Finally, the study may involve a limited number of bacterial isolates, which may not fully represent the diversity of *P. aeruginosa* strains present in poultry populations. Therefore, the generalization of the results should be made with caution.

1.9 Delimitations of the study

This study is specifically focused on evaluating the in vitro antimicrobial efficacy of *Punica granatum* peel extract against *Pseudomonas aeruginosa* isolated from poultry affected by airsacculitis. The research is limited to the use of peel extracts only and does not include other parts of the plant such as seeds, leaves, or bark.

The study is confined to a laboratory-based investigation and does not extend to in vivo testing in live poultry. Additionally, only selected concentrations of the extract will be tested to determine antimicrobial activity, and the study will focus on a single bacterial species rather than multiple pathogens associated with poultry respiratory diseases.

Furthermore, the study will utilize standard microbiological methods for isolation, identification, and antimicrobial testing, and will not incorporate advanced molecular or genomic analyses. The research is also limited to samples obtained within the Harare region in Zimbabwe from late February to early May, which defines the scope of the findings.

Chapter 2

Literature review

2.1 Airsacculitis in poultry

Airsacculitis is a respiratory condition usually seen in poultry and is characterized by the inflammation of the air sacs. This disease often appears as part of a respiratory disease complex which is frequently associated with bacterial infections. These infections can either be primary infections, or secondary infections (Glisson, 1998; Barnes et al., 2008).

Airsacculitis presents a significant hazard which warrants huge concern in poultry production due to its negative impact on bird health, productivity, and produce quality. The observable symptoms of airsacculitis are difficulty in breathing of the chickens, a discharge from the nose, coughing, sneezing, and reduced feeding. In severe cases, the affected birds may exhibit an observable weakness, weight loss, and increased number of deaths. Pathologically, the condition is characterized by the thickening and cloudiness of the air sacs, with the presence of fibrinous or cheese-like fluids exudated from the birds from areas like the pores (Jordan & Pattison, 1996).. These tears and lesions often lead to rejection of slaughtered poultry at processing plants and standardization bodies, resulting in substantial economic losses to the poultry industry (Barnes et al., 2008).

Airsacculitis is commonly associated with bacterial agents such as *Escherichia coli*, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, and *Pseudomonas aeruginosa* (Glisson, 1998). Environmental factors which can also contribute to the problem include poor ventilation, high ammonia levels, overcrowding, and inadequate biosecurity (Kabir, 2010). These factors predispose birds to infection by compromising their respiratory defenses. Opportunistic

pathogens may then infect the respiratory tract, leading to inflammation of the air sacs (Kabir, 2010).

Due to the many factors which lead to the rise of airsacculitis and its frequent association with opportunistic bacterial pathogens, effective control and treatment remain challenging. The increasing prevalence of antimicrobial resistance among poultry pathogens has further complicated disease management, highlighting the need for alternative therapeutic approaches.An example of a diseased chicken is shown in figure 2.1 below.



Figure 2.1 Diseased chicken. Credit: https://cs-tf.com/wp-content/uploads/2020/03/sick-chicken-symptoms-1024x695.jpg

2.2 *Pseudomonas aeruginosa* as a poultry pathogen

Pseudomonas aeruginosa is a Gram-negative, aerobic, rod-shaped bacterium usually found in the environment, particularly in soil, water, and in organic matter. It is widely regarded as an opportunistic pathogen capable of causing infections in both humans and animals. In poultry, *P. aeruginosa* has been observed in a wide range of disease conditions, especially when the birds are subjected to stress or in conditions where the immune system is compromised (Gellatly & Hancock, 2013).

In poultry production, *P. aeruginosa* commonly acts as a secondary pathogen, infecting the respiratory tract following primary infections or other environmental factors. This bacterial organism has been associated with respiratory diseases such as airsacculitis, pneumonia, and septicemia (Quinn et al., 2011). Important sources of infection which facilitate the spread of the pathogen within poultry flocks include contaminated water, litter, infected equipment, and unsanitary hatchery environments (Jawetz et al., 2016).

The pathogenicity of *P. aeruginosa* is attributed to several virulence factors, including exotoxins, proteases, and the ability to form biofilms (Gellatly and Hancock, 2013). The biofilm formation of *P. aeruginosa* enhances bacterial survival by protecting the organism from host immune responses and antimicrobial agents (Costerton et al., 1999). This feature contributes to the persistence of *P. aeruginos*a infections in poultry and complicates treatment efforts.

In cases of airsacculitis, *P. aeruginosa* colonizes the respiratory epithelium of birds and triggers inflammatory responses that may result in respiratory disease and septicemia (Campbell, 1986). The infection frequently produces lesions such as airsacculitis and fibrinous exudate accumulation within the air sacs, impairing respiratory function (Glisson 1998; Barnes et al., 2008). Young chicks and birds raised under poor hygienic conditions are particularly susceptible to infection due to stress and environmental contamination (AAAP, Avian Disease Manual, 2019).

The presence of *P. aeruginosa* in poultry is of considerable concern due to its intrinsic resistance to multiple antimicrobial agents, including commonly used antibiotics such as ciprofloxacin (Breidenstein et al., 2011; Lister et al., 2009). Studies have reported that many isolates exhibit

multidrug-resistant characteristics, which complicates the treatment of infections in poultry production systems (Lister et al., 2009; Gellatly and Hancock, 2013). Furthermore, the bacterium possesses numerous virulence factors and resistance genes that enhance its pathogenicity and survival in the environment (Breidenstein et al., 2011; Pang et al., 2019). As a result, increasing attention has been directed toward the development of alternative antimicrobial strategies, such as nanoparticle-based therapies, to control infections caused by *P. aeruginosa* (Pang et al., 2019).

2.3 Antibiotic resistance in *Pseudomonas aeruginosa*

Antibiotic resistance has become a major global public health and veterinary concern affecting both human and animal medicine (Van Boeckel et al., 2015). In poultry production, widespread antibiotic use contributes to the development of resistant bacterial strains (Marshall & Levy, 2011).

P. aeruginosa is particularly problematic due to its intrinsic and acquired resistance mechanisms to multiple antibiotic classes (Breidenstein et al., 2011). Examples of these, are the resistance to β-lactam antibiotics which occurs through β-lactamase production and porin loss (Lister et al., 2009), while resistance to aminoglycosides is mediated by aminoglycoside-modifying enzymes and efflux pumps (Poole, 2005).

Fluoroquinolone resistance arises from mutations in DNA gyrase and topoisomerase IV (Drlica & Zhao, 1997). Furthermore, the organism's ability to form biofilms and its low outer membrane permeability further enhances its antimicrobial resistance (Costerton et al., 1999; Lambert, 2002).

As a result, there is an increasing interest being directed toward alternative antimicrobial agents, particularly plant-derived compounds with bioactive properties like phytochemicals, which include flavonoids, tannins and phenols (Cowan, 1999).

2.4 Medicinal plants as alternative antimicrobial agents

For centuries, medicinal plants have been used in traditional medicine for the treatment of infectious diseases in Zimbabwe, especially by traditional healers (Maroyi, 2013). Examples of such plants and treatments used in Zimbabwe include *Lippia javanica* (Zumbani), whose leaves are traditionally used to treat respiratory infections, fever, and gastrointestinal disorders. Phytochemical analyses conducted by researchers locally have revealed the presence of flavonoids, alkaloids, and essential oils with demonstrated antibacterial activity against pathogens such as *S. aureus* and *E. coli* (Maroyi, 2017). Its essential oil fractions have also shown inhibitory effects on Gram-negative bacteria, supporting its use as a natural antimicrobial agent.

Another medicinal plant species used is *Warburgia salutaris* (Muranga), which is a medicinal tree indigenous to Zimbabwe. Its bark extracts are traditionally used to treat coughs, tuberculosis, and bacterial infections. Laboratory investigations have confirmed strong antimicrobial activity attributed to sesquiterpenoid compounds such as waburganal, which exhibit bactericidal effects against a range of pathogens (van Vuuren & Viljoen, 2011).

Aloe vera (Gavakava) and other related indigenous Aloe species are also widely used in Zimbabwean traditional medicine. Aloe gel and the plant's leaf extracts are applied to wounds and infections due to their antibacterial and anti-inflammatory properties. Studies have demonstrated inhibitory activity against bacteria like *P. aeruginosa* and other opportunistic pathogens. These properties are linked to anthraquinones and phenolic compounds which are present in the plant (Cock, 2008).

The antimicrobial properties of medicinal plants are primarily attributed to phytochemicals such as tannins, flavonoids, phenolic acids, alkaloids, and essential oils (Cowan, 1999). These compounds exert antimicrobial effects through mechanisms including disruption of bacterial cell membranes, inhibition of enzymatic activity, interference with nucleic acid synthesis, and suppression of biofilm formation (Burt, 2004; Nabavi et al., 2015).

The presence of multiple bioactive compounds in plant extracts may also reduce the likelihood of bacteria developing resistance compared with conventional single-compound antibiotics (Davies & Davies, 2010). In poultry production systems, plant-derived antimicrobials have gained increasing attention due to their availability, cost-effectiveness, biodegradability, and reduced risk of antibiotic residues in animal products (Windisch et al., 2008).

Numerous studies have demonstrated the inhibitory effects of plant extracts against poultry pathogens such as *E. coli*, *Salmonella* species, and *S. aureus* (Olasupo et al., 2003; Dorman & Deans, 2000). However, factors such as extraction method, plant part used, and extract concentration can significantly influence antimicrobial efficacy, highlighting the need for detailed evaluation of specific plant species and preparations (Harborne, 1998).

2.5 *Punica granatum* (Pomegranate)

2.5.1 Botanical description

Punica granatum, commonly known as pomegranate, is a fruit-bearing deciduous shrub or small tree belonging to the Lythraceae family. The plant is widely cultivated in tropical and subtropical regions and has long been valued for its nutritional and medicinal properties (Jurenka, 2008). Various parts of the plant, including the fruit, seeds, bark, leaves, and peel, have traditionally been used in the treatment of numerous ailments such as, inflammation, and gastrointestinal disorders (Viuda-Martos et al., 2010). In recent years, pomegranate cultivation has expanded in several regions of the world, including Zimbabwe, due to its economic and medicinal importance.

The pomegranate fruit is composed of an outer leathery peel that encloses numerous seeds surrounded by juicy arils. The peel, which constitutes nearly 30–40% of the fruit weight, is often discarded as agricultural waste during fruit processing (Fischer et al., 2011). However, several studies have demonstrated that the peel contains higher concentrations of bioactive compounds

such as polyphenols, tannins, and flavonoids compared to other parts of the fruit (Ismail et al., 2012). These compounds are responsible for the strong antioxidant and antimicrobial properties observed in pomegranate peel extracts. An example of a *P. granatum* tree is shown in figure 2.2 below



Figure 2.2 Pomegranate tree. Credit: Tadiwanashe Mhashu

Due to its rich phytochemical composition, pomegranate peel has attracted significant scientific interest as a potential source of natural antimicrobial agents. Studies have reported that extracts obtained from the peel exhibit inhibitory activity against a wide range of bacterial pathogens, including *E. coli*, *S. aureus*, and *Salmonella* species (Al-Zoreky, 2009). Consequently, pomegranate peel is increasingly being investigated as a natural alternative to synthetic antimicrobial agents in food preservation and animal health management.

2.5.2 Phytochemical composition of *Punica granatum* peel

Several studies have reported that *P. granatum* peel contains high concentrations of tannins, flavonoids, phenolic compounds, and alkaloids, which contribute to its biological activity (Jurenka, 2008; Viuda-Martos et al., 2010). Among these compounds, hydrolysable tannins such

as punicalagin and ellagic acid are considered the major bioactive constituents responsible for many of the pharmacological properties of pomegranate peel (Fischer et al., 2011). These compounds are widely recognized for their strong antioxidant and antimicrobial activities.

Phytochemicals present in pomegranate peel exert antimicrobial effects through several mechanisms. Tannins are known to precipitate proteins and disrupt microbial cell membranes, thereby inhibiting bacterial growth (Cowan, 1999). Similarly, flavonoids and phenolic compounds can interfere with bacterial enzyme systems, inhibit nucleic acid synthesis, and disrupt cellular metabolism (Daglia, 2012). Due to the high concentration of these bioactive compounds, pomegranate peel has been widely investigated as a promising natural source of antimicrobial agents for controlling pathogenic microorganisms (Xu et al., 2024).

2.5.3 Antimicrobial activity of *Punica granatum* peel extract

Although Gram-negative bacteria such as *P. aeruginosa* generally exhibit higher resistance to plant extracts due to the protective nature of their outer membrane, several studies have reported that pomegranate peel extract can still exert inhibitory effects against such organisms (Machado et al., 2003; Ismail et al., 2012). The presence of multiple bioactive compounds within the peel extract may produce a synergistic antimicrobial effect, thereby enhancing its activity against resistant bacterial strains.

The antimicrobial effectiveness of *P. granatum* peel extract has also been shown to depend on several factors, including the extraction solvent used, extract concentration, and the bacterial strain tested (Dahham et al., 2010). Despite growing evidence supporting the antimicrobial potential of pomegranate peel, relatively few studies have specifically examined its activity against *P. aeruginosa* isolated from poultry, particularly in association with respiratory infections such as airsacculitis. Therefore, further investigation is necessary to evaluate the effectiveness of pomegranate peel extracts against this pathogen and to explore their potential application as alternative antimicrobial agents in poultry disease management.

Chapter 3

Materials and methods

3.1 Collection and preparation of plant material

Fresh *P. granatum* (pomegranate) fruits were collected from locally available garden trees within the study area as shown in figure 3.1 below. Fresh plant material was used in order to ensure the preservation of naturally occurring bioactive compounds, since degradation may occur in aged or improperly stored samples (Azwanida, 2015).



Figure 3.1 Freshly collected *Punica granatum* fruits.

After collection, the fruits were thoroughly washed under running tap water in order to remove things such as dirt, dust, and other environmental contaminants. The fruits were then cut using a clean sterile knife in order to separate the peels from the seeds and the pulp. Only the peels were kept for further processing, as they are known to contain high concentrations of phytochemicals

such as phenols, tannins, and flavonoids, which are associated with antimicrobial activity (Viuda-Martos, Fernández-López, and Pérez-Álvarez, 2010).

The separated peels were cut into smaller pieces and were then spread evenly on clean trays followed by being air-dried at room temperature for one week. The drying was done in a well-aerated environment away from direct sunlight to prevent the degradation of heat and light sensitive phytochemicals. Controlled drying conditions were important in maintaining the integrity and effectiveness of plant-derived compounds (Sasidharan et al., 2011).

Completion of the peel drying was confirmed when the peels became hard and brittle. The dried peels were then ground into a fine powder using a clean mortar and pestle as shown in figure 3.2 below. This process increased the surface area of the plant material, thereby enhancing solvent penetration and improving the efficiency of extraction of bioactive constituents (Azwanida, 2015).

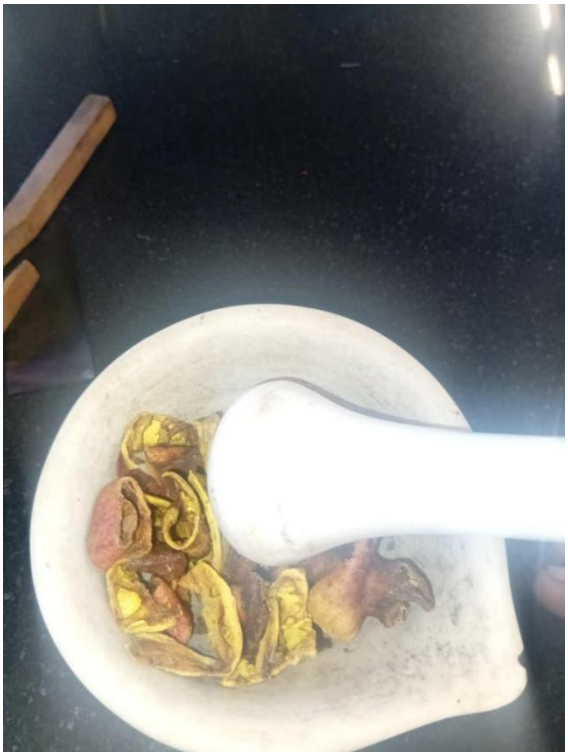


Figure 3.2 Grinding of the peels.

The resulting powdered material was then stored in clean, dry, airtight containers at room temperature waiting for the following steps.

3.2 Preparation of ethanol extract

The extraction of bioactive compounds from the powdered *P. granatum* peels was carried out using ethanol as the solvent. Ethanol was used because it is widely used in plant extraction due to its ability to dissolve a broad spectrum of phytochemicals, including phenols, flavonoids, and tannins, while remaining relatively safe and cost-effective (Azwanida, 2015; Sasidharan et al., 2011).

A 50% (v/v) ethanol solution was prepared by diluting 416.7 mL of 60% ethanol with 83.3 mL of distilled water, thus obtaining a 500mL 50% ethanol stock solution. The use of hydroethanolic solvent systems is well documented because it enhances extraction efficiency, due to the presence of water which improves the solubility of polar compounds while ethanol facilitates the extraction of moderately non-polar constituents (Do et al., 2014).

For the extraction process, 25 grams of the powdered pomegranate peel were weighed using an electronic balance as shown in figure 3.3 below and then transferred into a clean tray.



Figure 3.3. Weighing of powdered pomegranate peels.

All 25g of the ground powder was then added to the flask containing the 500mL 50% ethanol stock solution, thus maintaining a 1:20 (w/v) solid-to-solvent ratio. This ratio was used because it is commonly employed in plant extraction studies in order to ensure sufficient solvent volume for effective penetration and solubilization of phytochemicals (Azwanida, 2015).

The flask was then covered with aluminum foil to minimize contamination and solvent evaporation, and the mixture was left to stand in a dark cupboard at room temperature for 48 hours.

During this period, the mixture was periodically stirred in order to enhance the diffusion of bioactive compounds from the plant matrix into the solvent. This method was used because cold maceration is a simple yet effective extraction technique that allows for efficient recovery of thermolabile compounds without the need for heat (Sasidharan et al., 2011).

After 48 hours, the mixture was filtered using Whatman No. 1 filter paper in order to separate the liquid extract from the solid plant residues as shown in the images below. The filtrate, containing the dissolved phytochemicals, was collected into a clean beaker. This process is shown in figure 3.4, 3.5 and 3.6 below.



Figure 3.4 Getting ready to filter the solution after unwrapping the foil from the flask with the ethanolic extract.



Figure 3.5 Filtering the extract.



Figure 3.6 Collecting the filtrate.

The filtrate was then poured into sterile 250 mL beakers and was left uncovered at room temperature inside an evaporating cabinet for 5 days in order to allow complete evaporation of the ethanol as shown in figure 3.7 below. This process resulted in the formation of a concentrated crude extract. This was done because air evaporation at room temperature helps preserve heat-sensitive compounds that might otherwise degrade under high-temperature drying methods (Azwanida, 2015).



Figure 3.7 Evaporating the ethanol.

A jelly-like crude extract was obtained after the evaporation was done, which was weighed on a balance as shown in figure 3.8 below, and was found to weigh 14.6g.

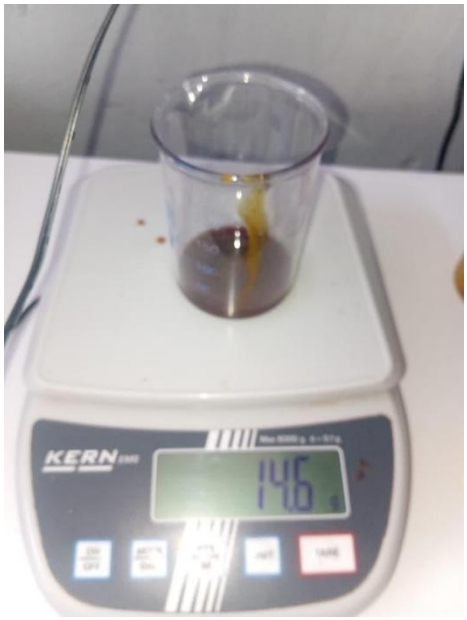


Figure 3.8 Weighing of the crude extract.

In order to re suspend the extract, as well as to prepare a 200mg/mL stock solution, it was added with distilled water until it reached a total volume of 73mL followed by shaking hard for 2 to 3 minutes, thereby creating a 50% EtOH extract, 200mg/mL stock solution which is shown in figure 3.9 below.

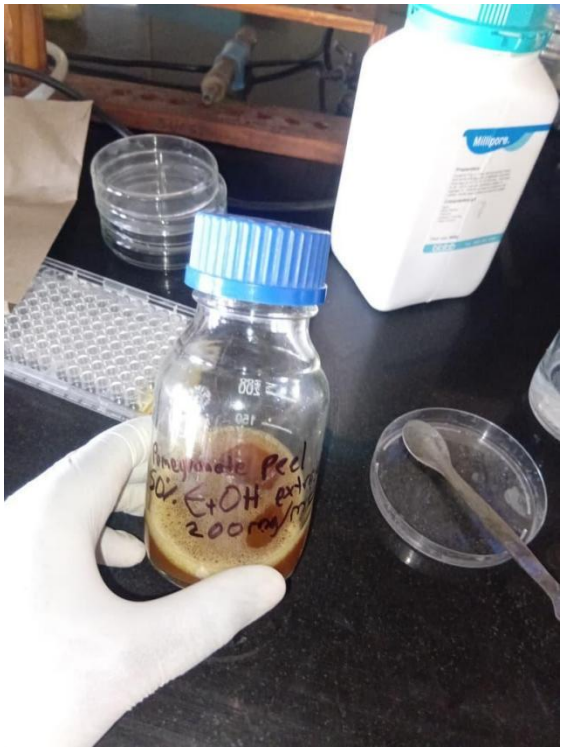


Figure 3.9 Extract stock solution.

3.3 Phytochemical screening for flavonoids, phenols, and tannins

Phytochemical screening of the *P.granatum* peel extract was then done in order to qualitatively determine the presence of key bioactive compounds, specifically flavonoids, phenols, and tannins. These compounds are widely recognized for their antimicrobial properties and are believed to greatly contribute to the inhibitory effects of plant extracts against pathogenic microorganisms (Harborne, 1998; Trease and Evans, 2009).

3.3.1 Test for flavonoids (alkaline reagent test)

The presence of flavonoids in the crude extract was determined using the alkaline reagent test, a standard qualitative method described in phytochemical analysis (Sofowora, 2008). In this procedure, a small quantity of the pomegranate peel extract was treated with a few drops of 10% sodium hydroxide (NaOH) solution.

The 10% NaOH solution was prepared by adding 20g of NaOH pellets to 200mL of distilled water.

The formation of an intense yellow coloration showed the presence of flavonoids. To confirm the result, dilute hydrochloric acid (HCl) was added to the mixture. The disappearance of the yellow color upon the addition of the HCL further confirmed the presence of flavonoids in the extract. This reaction occurs due to the ionization of flavonoid compounds under alkaline conditions, which is reversed when the medium becomes acidic (Harborne, 1998).

3.3.2 Test for phenols and tannins (ferric chloride test)

The presence of phenolic compounds and tannins was determined using the ferric chloride ($FeCl_3$) test, a widely accepted qualitative method for detecting phenolic hydroxyl groups (Trease and Evans, 2009).

In this test, 1–2 mL of the extract solution was placed in a test tube, followed by the addition of 2–5 drops of 5% ferric chloride solution.

The 5% Ferric chloride solution was prepared adding 5g of Ferric chloride hexahydrate powder to 100mL of distilled water, which was stirred until the powder was fully dissolved.

The development of a color change from blue-black to dark green indicated the presence of phenols and tannins. The intensity of the coloration provided a rough indication of the high concentration of these compounds within the extract.

This reaction is based on the formation of complexes between ferric ions (Fe^{3+}) and phenolic compounds, resulting in characteristic color changes (Sofowora, 2008).

3.4 Preparation of bacterial culture

A pure culture of *P. aeruginosa* was obtained and used for this study. Since the organism had already been isolated and identified prior to this experiment, no isolation procedures were carried out in this phase. The use of a pre-identified pure culture ensured reliability and consistency in the experimental results. The pure culture is shown in figure 3.10 below.



Figure 3.10 Pure *P. aeruginosa* culture.

The bacterial culture was first sub-cultured onto sterile nutrient agar plates to obtain fresh colonies. The inoculated plates were incubated at 37°C for 24 hours under aerobic conditions in order to promote optimal bacterial growth, as *P. aeruginosa* thrives well at this temperature (Cheesbrough, 2006).

After the incubation, well-isolated colonies were observed on the agar surface. A loopful of these colonies was carefully picked using a sterile inoculating loop and then transferred into a test tube containing a normal sterile saline solution. The bacterial suspension was then mixed thoroughly in order to achieve a uniform distribution of cells.

In order to standardize the bacterial inoculum, the turbidity of the suspension was adjusted to match the 0.5 McFarland standard which is shown in figure 3.11 below. This standard corresponds to an approximate bacterial density of 1.5×10^8 colony-forming units per milliliter (CFU/mL), which is essential for ensuring reproducibility and comparability of antimicrobial susceptibility results (CLSI, 2018).

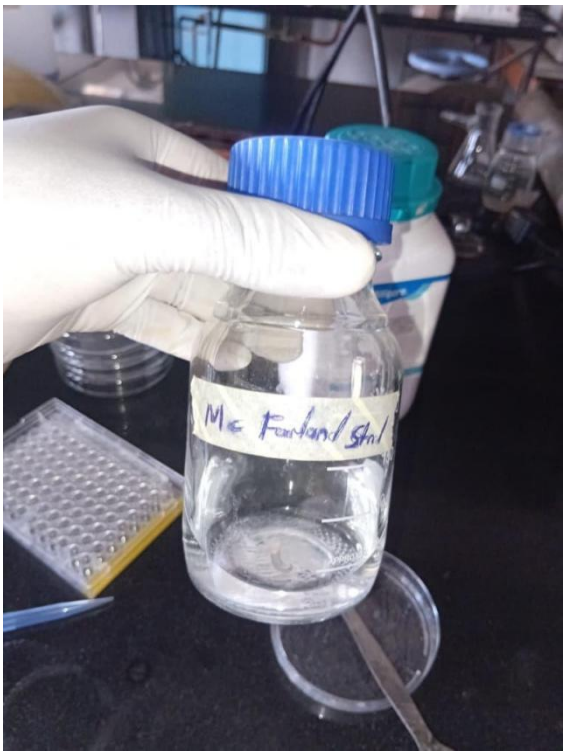


Figure 3.11 the 0.5 McFarland standard.

The adjustment was carried out visually by comparing the turbidity of the bacterial suspension to that of the McFarland standard against a white background with contrasting black lines manually. If the suspension appeared too turbid, it was diluted with sterile saline; if too light, more bacterial colonies were added until the desired turbidity was achieved.

The standardized bacterial suspension was used immediately for inoculation in antimicrobial susceptibility testing in order to prevent changes in cell density that could affect the outcome of the experiment.

3.5 Determination of antibacterial activity (agar well diffusion method)

The antibacterial activity of the *P. granatum peel extract* against *P. aeruginosa* was evaluated using the agar well diffusion method. This method is widely used for assessing the antimicrobial potential of plant extracts due to its simplicity, reproducibility, and ability to demonstrate clear zones of inhibition (Perez, Pauli, & Bazerque, 1990; Balouiri, Sadiki, & Ibnsouda, 2016).

Sterile Nutrient Agar (NA) plates were prepared according to the manufacturer's instructions. Nutrient agar was used due to the absence of Mueller-Hinton Agar (MHA) in the lab. And as a substitute, NA was selected because although it cannot match the efficiency of MHA, it can achieve some results, although those results might need to be interpreted in a cautious manner as they can have both positive and negative divergences (Ogofure et al., 2026).

Once the agar had solidified, each plate was inoculated with the standardized 0.5 McFarland suspension of *P. aeruginosa*. A sterile cotton swab was dipped into the bacterial suspension, and then excess fluid was removed by pressing against the inner wall of the test tube. The swab was then evenly spread across the entire surface of the agar plate. This process was repeated in three directions in order to ensure the formation of a uniform bacterial lawn.

After inoculation, pipette tips were used to punch uniform wells (about 6 mm in diameter) into the agar as shown in figure 3.12 below.

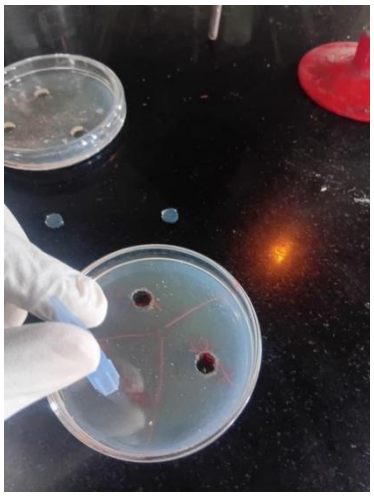


Figure 3.12 Creation of agar wells.

The wells were carefully labeled to correspond with different extract concentrations and control treatments. The different extract concentrations prepared by the direct dilution of the 50% EtOH extract, 200mg/mL stock solution into 100mg/mL, 50mg/mL, 25mg/mL, 12.5mg/mL and 6.25mg/mL solutions. Using a micropipette, a fixed volume of 100 µL of the prepared extract concentrations were dispensed into each well.

In order to ensure validity and reliability of the results, both positive and negative controls were included. Ciprofloxacin as a standard antibiotic, was used as the positive control in order to compare the effectiveness of the plant extract against a known antimicrobial agent. Sterile distilled water was used as a negative control.

The inoculated plates were allowed to stand at room temperature for approximately 30 minutes in order to allow for the pre-diffusion of the extract into the agar. The plates were then incubated at 37°C for 24 hours.

After incubation, the plates were examined for zones of inhibition, which appeared as clear areas surrounding the wells where bacterial growth had been suppressed. The diameter of each zone of inhibition was measured in millimeters using a dial caliper. These measurements were used to observe the antibacterial efficacy of the extract, with larger zones indicating stronger antimicrobial activity

3.6 Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the *P. granatum* peel extract against *P. aeruginosa* was determined in order to find the lowest concentration of the extract capable of inhibiting visible bacterial growth. Determining the MIC provides a more accurate evaluation of antimicrobial potency compared to general zone of inhibition measurements (Andrews, 2001).

A 96 well-plate was used as shown in figure 3.13 below with a goal to test from 100mg/mL concentration of the extract down to 0.39mg/mL concentration. This was done in order to provide a broader range for assessing concentration-dependent antimicrobial activity.



Figure 3.13 Conducting MIC.

For the plate setup, column 1 was the sterility control with wells loaded with 100 µL Nutrient Broth (NB) only. Column 2 was the growth control with wells loaded with 100 µL NB + 100 µL bacteria inoculum.

The bacterial inoculum was prepared and standardized using the same procedure described in section 3.5 followed by diluting it to 1:100 NB which was done by adding 100 µL of the 0.5 McFarland suspension to 9.9 mL of sterile NB.

Column 3-11 were the test wells.

For the dilution steps, 100 µL NB was added to wells in column 3. 200 µL of the 200 mg/mL extract was added to well A3 creating a 100 mg/mL concentration. 100 µL was then transferred from A3 to B3 creating a concentration of 50 mg/mL. There was continued transferring of 100 µL down the column resulting in 25 mg/mL, 12.6 mg/mL, 6.25 mg/mL, 3.125 mg/mL, 1.56 mg/mL, 0.78 mg/mL and 0.39 mg/mL concentrations.

100 µL was removed from the last well so that all wells have 100 µL. These steps were repeated across wells 4-11.

100 µL bacterial inoculum was then added to all test wells + growth control creating a final volume of 200 µL per well.

The plates were incubated at 37°C for 24 hours. After incubation, the plates were observed visually for turbidity in order to determine the MIC. The Growth control column was used as a reference for visualizing what cloudy growth looked like. The sterile column was used as a reference for determining what a clear no growth looked like.

The plate was read over a sheet with black lines with a light source underneath it. The plate was looked straight down at. If the black lines could be observed through the brown liquid, it indicated no growth. And if the brown liquid looked hazy, and milky, with badly visible lines compared to the sterile column, it would be determined as cloudy with bacterial growth.

3.7 Data analysis

The data obtained from the antimicrobial assays were carefully recorded, organized, and analyzed in order to evaluate the efficacy of *P. granatum* peel extract against *P. aeruginosa.* The primary data obtained in this study consisted of the diameters of zones of inhibition (measured in millimeters) for each extract concentration, as well as for the positive and negative controls. It also included visual turbidity data obtained from the MIC.

All experiments in the agar well diffusion were carried out in replicates in order to ensure the reliability and accuracy of the results. The measured zones of inhibition for each concentration were recorded and expressed as mean values ± standard deviation. This approach helped to minimize experimental error and it also provided a more accurate representation of the antimicrobial activity of the extract (Taylor, 1997).

The antibacterial activity of the extract was evaluated by comparing the mean zone of inhibition values across the different concentrations. Larger zones of inhibition were interpreted as indicating stronger antimicrobial activity. The effectiveness of the extract was also compared with that of the standard antibiotic Ciprofloxacin, which served as a positive control, to assess the relative potency of the plant extract.

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the extract that produced no visible turbidity in the well. This value was used to assess the minimum effective dose of the extract.

Where applicable, results were presented using tables and graphical representations such as bar charts to allow for clear visualization and comparison of antibacterial activity across different concentrations. Graphical analysis also helped to illustrate trends, such as the relationship between extract concentration and antimicrobial effectiveness.

All data analysis procedures followed standard approaches commonly used in microbiological and experimental research to ensure clarity, accuracy, and reproducibility of results (Gomez & Gomez, 1984).

Chapter 4

Results

4.1 Introduction

This chapter shows the results obtained from the investigation of the antimicrobial activity of *Punica granatum* peel extract against *Pseudomonas aeruginosa.* These results range from phytochemical screening, MIC, and agar well diffusion assay. They are presented in tables and bar graphs, as well as described accordingly.

The aim of the study was to evaluate whether or not the pomegranate peel extract demonstrated inhibitory effects against *P. aeruginosa*, as well as to evaluate the presence of the selected phytochemical compounds within the extract.

4.2 Phytochemical screening results

The ethanolic extract obtained from the *P. granatum* peels was tested for the presence of the selected bioactive compounds using qualitative phytochemical screening methods. The phytochemicals which were tested included flavonoids, phenols, and tannins.

The results of the tests revealed the presence of all the tested phytochemicals within the ethanolic extract. Observable color changes obtained during the tests confirmed the presence of these compounds within the extract as shown in Table 4.1 below.

Table 4.1 Phytochemicals detected in *Punica Granatum* Peel extract

Phytochemical	Test Used	Observation	Result
Flavonoids	Alkaline reagent test	Yellow coloration disappeared after addition of dilute HCL	Present (+)
Phenols	Ferric chloride test	Dark green coloration observed	Present (+)
Tannins	Ferric chloride test	Blue-black coloration observed	Present (+)

The alkaline reagent test resulted in a yellow coloration which disappeared after the addition of dilute hydrochloric acid, and thus confirming the presence of flavonoids within the extract. The ferric chloride test produced a blue-black coloration, showing the presence of phenolic compounds and tannins. The results from both tests are shown in in the image below.



Figure 3.14 Phytochemical screening test results.

By detecting these phytochemicals, it showed that *P. granatum* does indeed possess secondary metabolites that may contribute to its antimicrobial properties. Previous studies have shown that phenolic compounds, flavonoids, and tannins possess antibacterial activity through mechanisms such as the disruption of the cell membranes of microbial organisms, the inhibition of some bacterial enzymes, as well as some interference with the nutrient uptake in bacterial cells (Cowan, 1999; Daglia, 2012).

4.3 Antibacterial activity of *Punica granatum* peel extract against *Pseudomonas aeruginosa*

The ethanol extract of *P. granatum* peel was tested for its antimicrobial properties against *P. aeruginosa* using the agar well diffusion method. Its effectiveness was determined through measuring the diameters of the zones of inhibition formed around the wells containing different concentrations of the extract after incubating for an average of 24 hours at 37°C.

The experiment was carried out using three replicate samples labeled sample A, sample B and sample C in order to improve the reliability and reproducibility of the results. The inhibition zones obtained from each of the samples were measured in millimeters and then used to calculate the mean zone of inhibition and standard deviation for each concentration

The results obtained showed that the extract showed inhibitory activity against *P. aeruginosa* at all tested concentrations. The lowest concentration (6.25 mg/mL) produced only minimal inhibition. The size of the inhibition zones was observed to increase with increasing extract

concentration, showing a concentration-dependent antibacterial effect relationship. This is shown in table 4.2 below.

Table 4.2 Mean Zones of Inhibition Produced by Different Concentrations of *Punica granatum* Peel Extract Against *Pseudomonas aeruginosa*

Treatment	Concentration (mg/mL)	Sample A (mm)	Sample B (mm)	Sample C (mm)	Mean Zone of Inhibition (mm ± SD)
Punica granatum extract	100	15.1	15.3	14.9	15.1 ± 0.2
Punica granatum extract	50	11.2	10.8	11.4	11.13 ± 0.31
Punica granatum extract	25	7.9	8.7	8.3	8.3 ± 0.4
Punica granatum extract	12.5	4.8	5.4	5.6	5.27 ± 0.42
Punica granatum extract	6.25	2.6	2.2	3.1	2.63 ± 0.45
Ciprofloxacin	5 µg/mL	23.8	24.3	24.1	24.07 ± 0.25

(positive control)					
Sterile distilled water (negative control)	_	0	0	0	0.0 ± 0.0

The highest antibacterial activity of the extract was seen at a concentration of 100 mg/mL which produced a mean inhibition zone of 15.1 ± 0.2 mm. The following inhibition zones gradually decreased with decreasing extract concentration, with the lowest measurable activity being seen at 6.25 mg/mL.

The positive control, which is ciprofloxacin, produced the largest mean inhibition zone which was 24.07 ± 0.25 mm. This showed stronger antibacterial activity than the crude plant extract. And in contrast, the negative control, which was sterile distilled water, produced no inhibition zone which showed that the antibacterial activity observed was due to the phytochemicals present in the pomegranate peel extract.

A One-way ANOVA revealed a statistically significant difference among the treatment groups in mean zone of inhibition, $F_{(6, 14)} = 1923{,}30$, $_p < .001$. This indicates that the extract concentration significantly affected antibacterial activity.

A Tukey's Honestly Significant Difference (HSD) post-hoc test was conducted after the one-way ANOVA in order to identify the differences between the treatment means. The analysis revealed significant differences of $p < 0.001$ between all pairs of treatments. The mean zone of inhibition increased significantly with increasing concentration of *P. granatum* peel extract. Ciprofloxacin produced a significantly larger zone of inhibition than all extract concentrations, while the negative control exhibited significantly lower inhibition than all treatments.

4.4 Minimum inhibitory concentration (MIC) results

The minimum inhibitory concentration (MIC) of the ethanol extract of *P. granatum* peel against *P. aeruginosa* was determined as the lowest concentration of the extract which was capable of producing no visible turbidity due to bacterial growth after incubation as shown in figure 3.16 below.

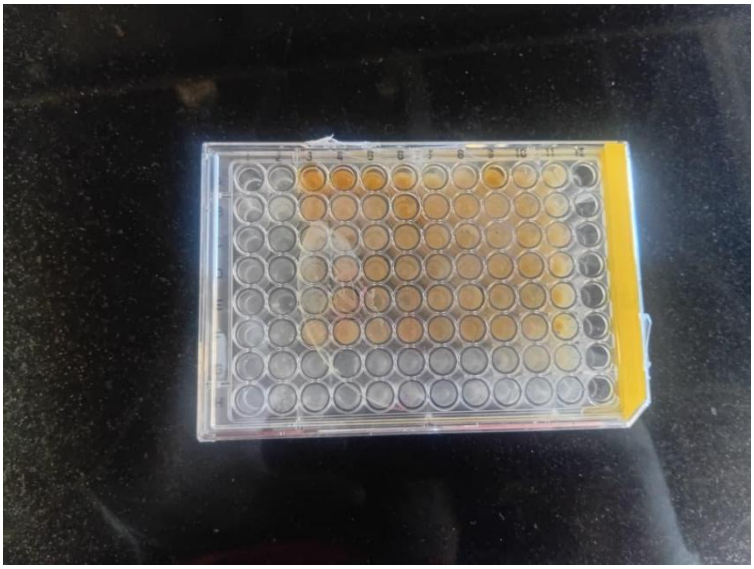


Figure 3.16 MIC plate after incubation.

The results that were obtained showed that the antibacterial activity of the extract decreased along with the decreasing concentration. Inhibitory activity was observed at concentrations ranging from 100 mg/mL to 25 mg/mL, and little visible inhibition was observed at the concentration of 12.5 mg/mL, whilst no inhibition was observed at 6.25 mg/mL and below. These results are shown in table 4.3 below.

Table 4.3 minimum inhibitory concentration (MIC) of *Punica granatum* peel extract against *Pseudomonas aeruginosa*

Extract concentration (mg/mL)	Observation after 24h	Interpretation

100	Clear wells	No visible growth
50	Clear wells	No visible growth
25	Clear wells	No visible growth
12.5	Slightly cloudy	Visible growth
6.25	Moderatel y cloudy	Visible growth
3.125	cloudy	Visible growth
1.56	cloudy	Visible growth
0.78	cloudy	Visible growth
0.39	cloudy	Visible growth
Growth control	Cloudy	Visible growth
Sterility control	Clear	No visible growth

The MIC of *P. granatum* peel extract against *P. aeruginosa* was determined to be 25mg/mL, which was defined as the lowest concentration with no visible turbidity.

On Nutrient Agar during well diffusion as noted in section 4.4, pomegranate peel extract produced a mean inhibition zone of 8.3 ± 0.4 mm at 25mg/mL. This weak zone diameter correlates with the MIC of 25mg/mL determined by broth microdilution, indicating that 25 mg/mL is required for complete growth inhibition.

4.5 Summary of findings

This study investigated the antimicrobial activity of the ethanolic extract obtained from *Punica granatum* peels against *P. aeruginosa*. Some qualitative phytochemical screenings of the extract that were conducted showed the presence of important bioactive compounds including flavonoids, phenols, and tannins within the extract.

The antibacterial susceptibility assay showed that the ethanol extract of *P. granatum* peel possessed inhibitory activity against *P. aeruginosa.* The extract produced measurable zones of inhibition at concentrations ranging from 100 mg/mL to 6.25 mg/mL, with antibacterial activity increasing as extract concentration increased. The highest antibacterial activity was observed at 100 mg/mL, whilst the lowest detectable inhibitory activity was observed at 6.25 mg/mL.

The positive control, ciprofloxacin showed the greatest antibacterial activity, whilst the negative control sterile distilled water showed no inhibitory effect. This showed that the antibacterial activity observed was due to the phytochemical constituents present in the pomegranate peel extract.

The Minimum inhibitory concentration (MIC) determination showed that the lowest concentration capable of inhibiting visible bacterial growth was 25 mg/mL and no inhibition of bacterial growth was observed below that.

In conclusion, these findings suggest that *P. granatum* peel extract possesses antibacterial properties against *P. aeruginosa*, likely due to the presence of phytochemical compounds such as flavonoids, tannins, and phenolic compounds identified during phytochemical screening.

Chapter 5

Discussion, summary, recommendations and conclusions

5.1 Discussion

5.1.1 Phytochemical screening

The qualitative phytochemical screening tests done on the ethanolic extract of *Punica granatum* peel showed the presence of flavonoids, phenolic compounds, and tannins. The detection of these phytochemicals supports the hypothesis that pomegranate peel contains bioactive secondary metabolites that may contribute to its antimicrobial activity against *P. aeruginosa*. The yellow coloration, which was seen in the alkaline reagent test, which disappeared upon the addition of dilute hydrochloric acid, confirmed the presence of flavonoids. The blue-black color changes seen during the ferric chloride tests confirmed the presence of tannins and phenolic compounds. These findings match with previous studies which have identified pomegranate peel as a rich source of polyphenolic compounds with antimicrobial properties (Viuda-Martos et al., 2010; Al-Zoreky, 2009).

The presence of flavonoids within the *P. granatum* peel extract was important because these compounds have been widely reported to possess antimicrobial effects against both Gram-positive and Gram-negative bacteria. Flavonoids show their antibacterial activity through several mechanisms, including the disruption of bacterial cell membranes, the inhibition of nucleic acid synthesis, the inhibition of energy metabolism, and their interference with bacterial enzyme systems (Cushnie & Lamb, 2011). Since *P. aeruginosa* possesses a complex outer membrane that contributes to its resistance to many antimicrobial agents, the ability of flavonoids to destabilize bacterial membranes may partially explain the inhibitory activity observed in this study.

Phenolic compounds were also detected in the extract. Phenolics are among the most extensively studied plant-derived antimicrobial compounds due to their ability to cause the denaturing of

proteins, the disruption of membranes, the inhibition of enzymes, as well as dealing oxidative damage within microbial cells (Daglia, 2012). The antimicrobial effectiveness of phenolic compounds has been associated with the presence of hydroxyl groups that interact with bacterial proteins and cell membranes, ultimately impairing cellular function. The detection of phenols in the present study therefore provides a biochemical basis for the antibacterial activity observed during both the agar well diffusion and minimum inhibitory concentration assays.

Tannins were also shown to be present in the extract. Tannins are high molecular weight polyphenolic compounds capable of binding to proteins and other macromolecules. Their antimicrobial activity is often credited to their ability to precipitate microbial proteins, inhibit extracellular microbial enzymes, deprive microorganisms of essential substrates, and disrupt cell membrane integrity (Cowan, 1999). Previous investigations have reported that tannins isolated from plant materials exhibit significant inhibitory effects against several pathogenic bacteria, including antibiotic-resistant strains. Therefore, their presence in the pomegranate peel extract may have contributed significantly to the observed antibacterial activity against *P. aeruginosa*.

The combined presence of flavonoids, phenols, and tannins shows that the antibacterial activity observed in this study was not likely due to a single phytochemical compound but rather due to a synergistic interaction among multiple bioactive constituents. Synergistic interactions between phytochemicals have been shown to enhance antimicrobial effectiveness through simultaneous action on different bacterial targets, thereby reducing the likelihood of bacterial adaptation and resistance (Wagner and Ulrich-Merzenich, 2009). This might explain why the crude extract demonstrated measurable antibacterial activity despite not being purified into individual active compounds.

The successful extraction of these phytochemicals also validates the extraction methodology employed in this study. The use of a 50% hydroethanolic solvent system was effective in extracting both polar and moderately non-polar compounds. This finding is in line with previous studies which reported that hydroethanolic solvents generally yield higher concentrations of phenolic compounds and flavonoids compared to water alone because they improve the solubility and recovery of diverse phytochemicals (Do et al., 2014).

Overall, the phytochemical screening results demonstrate that *P. granatum* peel contains important secondary metabolites associated with antimicrobial activity. The presence of

flavonoids, phenols, and tannins provides a scientific explanation for the antibacterial effects observed against *P. aeruginosa* and supports the potential use of pomegranate peel as a source of natural antimicrobial agents.

5.1.2 Antibacterial activity of *Punica granatum* peel extract against *Pseudomonas aeruginosa*

The agar well diffusion assay showed that the ethanolic extract of *P. granatum* peel possessed measurable antibacterial activity against *P. aeruginosa*. The results showed that the extract produced inhibition zones at all tested concentrations, with antibacterial activity increasing as extract concentration increased. The highest concentration tested at 100 mg/mL produced a mean inhibition zone of 15.1 ± 0.2 mm, while the lowest concentration 6.25 mg/mL produced the smallest measurable inhibition zone of 2.63 ± 0.45 mm. These findings showed that the antibacterial activity of the extract was concentration dependent.

The observed increase in inhibition zone diameter with increasing extract concentration suggests that higher concentrations contained greater quantities of bioactive compounds capable of suppressing bacterial growth. As the concentration of flavonoids, phenolic compounds, and tannins increases, more antimicrobial molecules diffuse into the agar medium and interact with bacterial cells, resulting in greater inhibition of microbial growth. Similar concentration-dependent antibacterial activity of pomegranate peel extracts has been reported in previous studies against various Gram-negative bacteria, including *P. aeruginosa* (Al-Zoreky, 2009; Duman et al., 2009).

The findings of this study are particularly significant because *P. aeruginosa* is widely recognized as one of the most difficult bacterial pathogens to control due to its intrinsic resistance mechanisms. The bacterium possesses an outer membrane with low permeability, multiple efflux pump systems, and the ability to form biofilms, all of which contribute to resistance against many antimicrobial agents (Pang et al., 2019). Despite these defense mechanisms, the pomegranate peel extract was still able to inhibit bacterial growth, indicating the presence of compounds capable of overcoming at least some of these resistance barriers.

Although antibacterial activity was observed, the extract produced smaller inhibition zones than ciprofloxacin, which generated a mean inhibition zone of 24.07 ± 0.25 mm. This result was expected because ciprofloxacin is a purified pharmaceutical antibiotic with a well-established mechanism of action targeting bacterial DNA gyrase and topoisomerase IV, resulting in highly efficient bacterial inhibition (Hooper and Jacoby, 2016). In contrast, the pomegranate peel extract was a crude preparation containing a mixture of active and inactive compounds. Consequently, the concentration of specific antibacterial constituents within the extract was considerably lower than that of the purified antibiotic. Nevertheless, the fact that the crude extract exhibited substantial antibacterial activity highlights its potential as a natural antimicrobial agent.

The absence of any inhibition zone in the negative control containing sterile distilled water confirmed that the observed antibacterial activity was solely due to the phytochemical constituents present in the extract and not the solvent or experimental conditions. This finding strengthens the validity of the study and supports the conclusion that the inhibitory effect resulted from the bioactive compounds extracted from the pomegranate peel.

The statistical analysis further reinforced these observations. The one-way analysis of variance (ANOVA) revealed a statistically significant difference among treatment groups ($F = 1923.30$, $p < 0.001$). This result showed that differences in extract concentration had a significant effect on antibacterial activity and that the variations observed in inhibition zones were unlikely to have occurred by chance. The statistical significance is important because it provides objective evidence that the extract concentration directly influenced the antibacterial response observed during the experiment.

Furthermore, the Tukey Honestly Significant Difference (HSD) post hoc test demonstrated significant differences between all treatment groups ($p < 0.001$). This finding confirms that each increase in concentration produced a meaningful increase in antibacterial activity. The progressive increase in inhibition zones across concentrations provides strong evidence of a dose-response relationship between the extract and *P. aeruginosa*. Such dose-dependent responses are frequently regarded as indicators of genuine pharmacological or antimicrobial activity because they demonstrate a predictable relationship between the amount of active substance present and the biological effect produced (Balouiri et al., 2016).

The relatively small standard deviation values observed across all treatment groups indicate a high degree of consistency among the experimental replicates. This suggests that the methodology used in this study, including bacterial inoculum standardization, preparation of extract concentrations, incubation conditions, and measurement procedures, produced reliable and reproducible results. The low variability between replicates increases confidence in the accuracy of the findings and supports the overall validity of the study.

The antibacterial activity observed in this investigation is likely attributable to the combined effects of flavonoids, phenolic compounds, and tannins identified during phytochemical screening. Phenolic compounds may disrupt bacterial membranes and interfere with enzyme systems, while tannins may precipitate proteins and inhibit bacterial metabolism. Flavonoids may further contribute by damaging bacterial cell walls, disrupting membrane permeability, and inhibiting nucleic acid synthesis (Cowan, 1999; Cushnie & Lamb, 2011; Daglia, 2012). The combined action of these phytochemicals may have produced a synergistic effect that enhanced the overall antibacterial activity of the extract.

The findings of this study are consistent with previous reports which have shown that pomegranate peel extracts exhibit antibacterial activity against a range of pathogenic microorganisms. Al-Zoreky (2009) reported that pomegranate peel extracts demonstrated significant inhibitory effects against several foodborne pathogens, while Duman et al. (2009) observed antibacterial activity against both Gram-positive and Gram-negative bacteria. The agreement between the present findings and those reported in earlier studies strengthens the evidence supporting the antimicrobial potential of *P. granatum* peel.

Overall, the agar well diffusion results demonstrate that *P. granatum* peel extract possesses significant antibacterial activity against *P. aeruginosa*. Although the activity was lower than that of ciprofloxacin, the extract exhibited a clear concentration-dependent inhibitory effect, supported by highly significant statistical analysis. These findings support the potential application of pomegranate peel as a natural source of antimicrobial compounds and contribute to the growing body of evidence supporting the use of medicinal plants in combating bacterial infections.

5.1.3 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) assay was conducted to determine the lowest concentration of *P. granatum* peel extract capable of preventing visible growth of *P. aeruginosa*. The results showed that the MIC of the ethanolic pomegranate peel extract was 25 mg/mL. This concentration was identified as the lowest extract concentration that produced clear wells with no visible turbidity following incubation, indicating complete inhibition of bacterial growth. Concentrations below 25 mg/mL exhibited varying degrees of turbidity, suggesting that bacterial growth was no longer fully suppressed.

The determination of the MIC provides important information regarding the antimicrobial potency of the extract. While agar well diffusion assays demonstrate antibacterial activity through the formation of inhibition zones, MIC testing offers a more precise quantitative assessment of antimicrobial effectiveness by identifying the minimum concentration required to inhibit bacterial growth (Andrews, 2001). Therefore, the MIC value obtained in this study serves as a critical indicator of the antibacterial strength of *P. granatum* peel extract against *P. aeruginosa*.

The MIC value of 25 mg/mL indicates that the extract exhibited measurable to moderate antibacterial activity against *P. aeruginosa*, particularly considering that a crude plant extract was evaluated rather than purified antimicrobial compounds. Although lower MIC values generally indicate stronger antimicrobial potency, the observed MIC is noteworthy considering that *P. aeruginosa* is inherently resistant to many antimicrobial agents due to its highly impermeable outer membrane, multidrug efflux pumps, and biofilm-forming capability (Pang et al., 2019). The ability of the pomegranate peel extract to completely inhibit bacterial growth at 25 mg/mL therefore demonstrates that the extract contains biologically active compounds capable of overcoming some of the organism's natural defense mechanisms.

The MIC findings also showed a strong correlation with the agar well diffusion results. At the MIC concentration of 25 mg/mL, the agar well diffusion assay produced a mean inhibition zone of 8.3 ± 0.4 mm. Although this inhibition zone was smaller than those observed at higher concentrations, it nevertheless demonstrated measurable antibacterial activity. The agreement between the two methods strengthens the reliability of the findings because both assays independently identified 25 mg/mL as a critical threshold at which effective bacterial inhibition

occurred. Such consistency between diffusion-based and broth dilution methods is often considered evidence of reliable antimicrobial activity (Balouiri et al., 2016).

The progressive increase in turbidity observed below the MIC concentration further supports the concentration-dependent antibacterial effect observed during the agar well diffusion assay. At concentrations of 12.5 mg/mL and below, visible bacterial growth was observed, indicating that the concentration of antimicrobial compounds was insufficient to completely suppress bacterial multiplication. This trend suggests that the antibacterial activity of the extract depends on the availability of adequate concentrations of active phytochemicals. As extract concentration decreases, the quantity of bioactive compounds available to interact with bacterial cells also decreases, resulting in reduced antimicrobial effectiveness.

The antimicrobial activity demonstrated at the MIC may be attributed to the flavonoids, phenolic compounds, and tannins identified during phytochemical screening. These compounds have been reported to interfere with microbial growth through multiple mechanisms. Phenolic compounds can disrupt bacterial cell membranes and alter membrane permeability, causing leakage of essential cellular components. Tannins may inhibit bacterial enzymes and precipitate microbial proteins, while flavonoids can interfere with nucleic acid synthesis and energy metabolism (Cowan, 1999; Cushnie & Lamb, 2011; Daglia, 2012). The simultaneous action of these phytochemicals may explain the ability of the extract to inhibit growth at concentrations as low as 25 mg/mL.

The MIC obtained in this study is generally consistent with findings from previous investigations involving pomegranate peel extracts. Numerous studies have reported that pomegranate peel extracts exhibit inhibitory effects against both Gram-positive and Gram-negative bacteria, although reported MIC values vary considerably depending on factors such as extraction solvent, extraction method, bacterial strain, geographical origin of plant material, and phytochemical composition (Al-Zoreky, 2009; Nuamsetti, Dechayuenyong, & Tantipaibulvut, 2012). Variations in MIC values among studies are therefore expected and do not necessarily indicate inconsistencies in antimicrobial effectiveness.

It is important to note that the extract evaluated in this study was a crude extract rather than a purified preparation. Crude extracts contain a mixture of active compounds together with inactive substances that may dilute the overall antimicrobial potency. Purification and isolation

of specific active constituents such as punicalagins, ellagic acid, and other polyphenolic compounds could potentially result in lower MIC values and enhanced antibacterial activity (Viuda-Martos et al., 2010). Therefore, the MIC reported in this study may underestimate the true antimicrobial potential of the most active compounds present within pomegranate peel.

The MIC results also have practical significance in the context of increasing antimicrobial resistance. The growing prevalence of multidrug-resistant *P. aeruginosa* strains has created a need for alternative antimicrobial agents derived from natural sources. The ability of pomegranate peel extract to inhibit bacterial growth at a relatively moderate concentration suggests that it may serve as a promising source of antibacterial compounds for future pharmaceutical, food preservation, or healthcare applications. However, further studies involving purification of active constituents, toxicity evaluation, and testing against clinical multidrug-resistant isolates would be required before practical applications can be developed.

Overall, the MIC results confirmed that *P. granatum* peel extract possesses genuine antibacterial activity against *P. aeruginosa*. The MIC value of 25 mg/mL supports the findings obtained from the agar well diffusion assay and provides quantitative evidence of antimicrobial potency. The results further suggest that the phytochemical constituents present within pomegranate peel contribute significantly to bacterial growth inhibition and support the potential of this plant material as a source of natural antimicrobial agents.

5.1.3 Implications of the study

The findings of this study have important scientific and practical implications regarding the use of *P. granatum* peel as a potential source of natural antimicrobial agents. The study successfully demonstrated the presence of flavonoids, phenolic compounds, and tannins within the ethanolic peel extract, while both the agar well diffusion assay and minimum inhibitory concentration assay confirmed antibacterial activity against *P. aeruginosa*. Collectively, these findings indicate that pomegranate peel contains biologically active compounds capable of inhibiting the growth of an important pathogenic bacterium.

One of the major implications of these findings is the potential utilization of pomegranate peel as an alternative source of antimicrobial compounds. The increasing prevalence of antimicrobial resistance has become a significant global public health challenge. *P. aeruginosa* is among the pathogens listed by the World Health Organization as a priority organism due to its capacity to

develop resistance to multiple classes of antibiotics (World Health Organization [WHO], 2024). The demonstration of antibacterial activity by pomegranate peel extract suggests that plant-derived compounds may contribute to the development of alternative therapeutic agents or adjunct treatments capable of supporting existing antimicrobial strategies.

The findings also contribute to the growing body of evidence supporting the medicinal value of agricultural waste products. Pomegranate peels are often discarded as waste following fruit consumption despite containing substantial concentrations of bioactive compounds. The results of this study suggest that these discarded peels may represent a valuable and inexpensive source of antimicrobial agents. This finding is particularly relevant in developing countries where access to expensive pharmaceutical products may be limited and where locally available medicinal plants can provide cost-effective alternatives for healthcare applications.

The concentration-dependent antibacterial activity observed in both the agar well diffusion assay and MIC determination further suggests that the antimicrobial efficacy of the extract is directly related to the concentration of phytochemical constituents present. This observation supports the hypothesis that flavonoids, phenolic compounds, and tannins are likely responsible for the inhibitory effects observed against *P. aeruginosa*. The relationship between phytochemical concentration and antibacterial activity also indicates the possibility of enhancing antimicrobial effectiveness through purification, fractionation, or concentration of the active compounds.

The findings additionally provide support for future investigations aimed at isolating specific phytochemical compounds responsible for antibacterial activity. Although the crude extract demonstrated measurable antimicrobial effects, it is possible that purification of individual compounds such as punicalagins, ellagic acid, gallotannins, and other polyphenols could yield stronger antibacterial activity than that observed in the present study. Such investigations may contribute to the discovery of novel antimicrobial compounds with potential pharmaceutical applications.

However, the implications of the findings must be interpreted while considering certain methodological limitations. One important limitation was the use of Nutrient Agar (NA) instead of Mueller-Hinton Agar (MHA) during the agar well diffusion assay. Mueller-Hinton Agar is internationally recognized as the standard medium for antimicrobial susceptibility testing because it possesses controlled levels of nutrients, starch content, pH, and agar depth that

promote consistent diffusion of antimicrobial agents throughout the medium (CLSI, 2024). In contrast, Nutrient Agar contains different nutrient compositions that may alter the diffusion characteristics of antimicrobial compounds.

The use of Nutrient Agar may therefore have introduced bias into the measured zone of inhibition diameters. Plant-derived phytochemicals diffuse through agar media at varying rates depending on their molecular size, polarity, and interaction with agar components. Because Nutrient Agar is richer in nutrients and differs in composition from Mueller-Hinton Agar, antimicrobial compounds may diffuse either more slowly or more rapidly than they would under standardized testing conditions. Consequently, the inhibition zones observed in this study may not perfectly represent the true antibacterial potency of the extract. The measured zones could have been underestimated if diffusion was restricted or overestimated if diffusion was enhanced by the medium composition (Ogofure et al., 2026).

Furthermore, results generated using Nutrient Agar may not be directly comparable with those reported in studies that employed Mueller-Hinton Agar according to Clinical and Laboratory Standards Institute (CLSI) guidelines. While the observed concentration-dependent trend and statistical significance remain valid, caution should be exercised when comparing the absolute inhibition zone diameters obtained in this study with published susceptibility data from other investigations. Therefore, the antibacterial activity demonstrated by the extract should be interpreted primarily as evidence of inhibitory potential rather than as a definitive measure of antimicrobial potency.

Despite this limitation, the consistency of the results across replicate experiments, the highly significant ANOVA findings, the significant Tukey HSD comparisons, and the agreement between the agar well diffusion and MIC assays strengthen confidence in the overall conclusions of the study. The MIC assay, in particular, is less dependent on diffusion properties than agar diffusion methods and therefore provides independent confirmation that the extract possesses genuine antibacterial activity against *P. aeruginosa*.

Overall, the findings indicate that *P. granatum* peel represents a promising source of natural antimicrobial compounds with activity against *P. aeruginosa*. Although additional studies employing standardized susceptibility testing media, phytochemical quantification, compound isolation, toxicity evaluation, and testing against clinical multidrug-resistant isolates are required,

the results provide a strong foundation for future research into the development of plant-based antimicrobial agents.

5.2 Summary

This study was conducted to evaluate the antimicrobial activity of ethanolic *P. granatum* (pomegranate) peel extract against *P. aeruginosa* and to determine the phytochemical constituents that may contribute to its antibacterial effects. The findings obtained from phytochemical screening, agar well diffusion assays, and minimum inhibitory concentration (MIC) determination collectively demonstrated that pomegranate peel possesses bioactive compounds capable of inhibiting the growth of *P. aeruginosa*.

The qualitative phytochemical analysis confirmed the presence of flavonoids, phenolic compounds, and tannins within the ethanolic peel extract. These phytochemicals are widely recognized for their antimicrobial properties and have been reported to inhibit bacterial growth through mechanisms such as disruption of cell membrane integrity, inhibition of essential enzymes, interference with nutrient uptake, and impairment of nucleic acid synthesis. The detection of these compounds provided a biochemical basis for the antibacterial activity observed during subsequent antimicrobial susceptibility testing.

The agar well diffusion assay demonstrated that the extract exhibited antibacterial activity against *P. aeruginosa* across all tested concentrations. The highest concentration of the extract (100 mg/mL) produced the largest mean zone of inhibition (15.1 ± 0.2 mm), while lower concentrations produced progressively smaller inhibition zones. This concentration-dependent response indicated that antibacterial activity increased as extract concentration increased. Statistical analysis using one-way ANOVA revealed a highly significant difference among treatment groups ($p < 0.001$), while Tukey's HSD post hoc analysis confirmed significant differences between individual concentrations. These findings provide strong evidence that the antibacterial activity of the extract was directly influenced by concentration.

The minimum inhibitory concentration assay further confirmed the antimicrobial effectiveness of the extract. The MIC was determined to be 25 mg/mL, representing the lowest concentration capable of completely inhibiting visible bacterial growth. The agreement between the MIC

results and agar well diffusion findings strengthened the reliability of the study by demonstrating consistent antibacterial activity through two independent testing methods.

Although ciprofloxacin exhibited greater antibacterial activity than the crude pomegranate peel extract, the extract nevertheless demonstrated substantial inhibitory effects against *P. aeruginosa*, a bacterium known for its intrinsic and acquired resistance mechanisms. This finding highlights the potential of pomegranate peel as a source of natural antimicrobial compounds that may contribute to future strategies aimed at addressing antimicrobial resistance.

However, the interpretation of the agar well diffusion results should be considered in light of certain methodological limitations. The use of Nutrient Agar instead of the standard Mueller-Hinton Agar may have influenced the diffusion of antimicrobial compounds and therefore affected inhibition zone diameters. Despite this limitation, the consistency of replicate measurements, the significant statistical findings, and the corroborating MIC results provide confidence that the antibacterial activity observed was genuine and attributable to the phytochemical constituents present in the extract.

5.3 Recommendations

Based on the findings obtained in this study, the following recommendations are proposed for future research and potential practical applications of *P. granatum* peel extract as a natural antimicrobial agent.

Firstly, future studies should use Mueller-Hinton Agar (MHA) instead of Nutrient Agar when conducting agar well diffusion susceptibility testing. MHA is the internationally accepted standard medium recommended by the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial susceptibility testing because it provides more consistent diffusion of antimicrobial agents and allows easier comparison of results with those reported in other studies. The use of MHA would improve the accuracy, reproducibility, and comparability of inhibition zone measurements.

Secondly, quantitative phytochemical analysis should be conducted to determine the exact concentrations of flavonoids, phenolic compounds, tannins, and other bioactive constituents present in *P. granatum* peel extract. While the present study qualitatively confirmed the presence of these compounds, quantification would allow researchers to establish relationships between

phytochemical concentration and antibacterial activity. Such information would improve understanding of which compounds contribute most significantly to antimicrobial effects.

Thirdly, future research should focus on the isolation, purification, and characterization of individual bioactive compounds from pomegranate peel. The extract used in this study was a crude extract containing both active and inactive constituents. Isolation of specific compounds such as punicalagins, ellagic acid, gallic acid, and other polyphenols may reveal stronger antibacterial activity than that observed with the crude extract and could facilitate the development of novel antimicrobial agents.

Fourthly, studies should investigate the antibacterial activity of *P. granatum* peel extract against a broader range of clinically important microorganisms. While the present study focused on *P. aeruginosa*, future investigations should include other Gram-negative and Gram-positive pathogens such as *E. coli*, *K. pneumoniae*, *S. aureus*, and *E. faecalis*. Such studies would provide a more comprehensive assessment of the antimicrobial spectrum of the extract.

Fifthly, future investigations should evaluate the effectiveness of pomegranate peel extract against multidrug-resistant clinical isolates. Antimicrobial resistance is an increasing global health concern, and determining whether the extract can inhibit resistant bacterial strains would provide valuable information regarding its potential therapeutic applications. Testing against resistant isolates obtained from hospital settings may offer insights into the practical relevance of the extract in combating antimicrobial resistance.

Sixthly, toxicity and safety studies should be conducted before any clinical or pharmaceutical applications are considered. Although pomegranate is generally regarded as safe for consumption, concentrated extracts may exhibit biological effects that require careful evaluation. Cytotoxicity studies, animal model experiments, and safety assessments should therefore be undertaken to establish safe dosage ranges and identify any potential adverse effects associated with prolonged exposure to the extract.

Seventhly, future studies should investigate the synergistic effects of combining *P. granatum* peel extract with conventional antibiotics. Previous studies have suggested that certain plant-derived compounds can enhance the effectiveness of antibiotics by disrupting bacterial defense mechanisms. Evaluating combinations of pomegranate peel extract with antibiotics such as

ciprofloxacin, gentamicin, or ceftazidime may reveal synergistic interactions capable of improving treatment outcomes while reducing antibiotic dosage requirements.

Eighthly, Future studies should determine the Minimum Bactericidal Concentration (MBC) of *P. granatum* peel extract against *P. aeruginosa*. While the present study established the Minimum Inhibitory Concentration, the MBC would provide additional information regarding whether the extract merely inhibits bacterial growth or is capable of killing bacterial cells. This distinction would improve understanding of the antimicrobial potential of the extract and its possible therapeutic applications.

Finally, optimization of extraction procedures should be explored to maximize the recovery of antimicrobial compounds from pomegranate peels. Factors such as solvent type, solvent concentration, extraction time, extraction temperature, and extraction method may significantly influence phytochemical yield and antimicrobial activity. Identifying optimal extraction conditions could improve the effectiveness of the extract and enhance its suitability for large-scale applications.

while the present study demonstrated that ethanolic *P. granatum* peel extract possesses significant antibacterial activity against *P. aeruginosa*, further investigations are required to standardize testing methods, identify active compounds, evaluate safety, and determine practical applications. Implementation of these recommendations would contribute to a deeper understanding of the antimicrobial potential of pomegranate peel and support the development of plant-based alternatives for managing bacterial infections.

5.4 Conclusions

In conclusion, the results of this study demonstrate that ethanolic extracts of *P. granatum* peel contain biologically active phytochemicals capable of inhibiting the growth of *P. aeruginosa*. The presence of flavonoids, phenols, and tannins, together with the concentration-dependent inhibition observed in both agar well diffusion and MIC assays, provide evidence supporting the antimicrobial potential of pomegranate peel. Although the extract was less effective than ciprofloxacin and certain methodological limitations were present, the findings indicate that pomegranate peel may serve as a valuable source of natural antimicrobial compounds worthy of further investigation and development.

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Appendices

Appendix A raw zone of inhibition data

Table A1. Raw inhibition zone measurements (mm) of *Punica granatum* peel extract against *Pseudomonas aeruginosa*

Treatment	Replicate 1 (mm)	Replicate 2 (mm)	Replicate 3 (mm)
100 mg/mL	15.1	15.3	14.9
50 mg/mL	11.2	10.8	11.4
25 mg/mL	7.9	8.7	8.3
12.5 mg/mL	4.8	5.4	5.6
6.25 mg/mL	2.6	2.2	3.1
Ciprofloxacin	23.8	24.3	24.1
Distilled Water	0.0	0.0	0.0

Appendix B phytochemical screening results

Table B1. Qualitative phytochemical screening of *Punica granatum* peel extract

Phytochemical	**Test Used**	**Observation**	**Result**
Flavonoids	NaOH test	Yellow coloration	Present
Phenols	Ferric chloride test	Dark green/ Blue-black coloration	Present
Tannins	Ferric chloride test	Blue-black coloration	Present

Appendix C anova output

Table C1. One-way ANOVA comparing mean zones of inhibition

Source	Df	F-value	p-value
Between Groups	6	1923.30	<0.001
Within Groups	14	-	-
Total	20	-	-

Appendix D Tukey HSD Post Hoc results

Table D1. Tukey HSD multiple comparison analysis of inhibition zones

Comparison	Mean Difference (mm)	p-value	Significant?
100 mg/mL vs 50 mg/mL	3.97	<0.001	Yes
100 mg/mL vs 25 mg/mL	6.80	<0.001	Yes
100 mg/mL vs 12.5 mg/mL	9.83	<0.001	Yes
100 mg/mL vs 6.25 mg/mL	12.47	<0.001	Yes
100 mg/mL vs Ciprofloxacin	8.97	<0.001	Yes
100 mg/mL vs Negative Control	15.10	<0.001	Yes
50 mg/mL vs 25 mg/mL	2.83	<0.001	Yes
50 mg/mL vs 12.5 mg/mL	5.87	<0.001	Yes
50 mg/mL vs 6.25 mg/mL	8.50	<0.001	Yes
50 mg/mL vs Ciprofloxacin	12.93	<0.001	Yes
50 mg/mL vs Negative Control	11.13	<0.001	Yes
25 mg/mL vs 12.5 mg/mL	3.03	<0.00	Yes

Comparison	Mean Difference (mm)	p-value	Significant?
25 mg/mL vs 6.25 mg/mL	5.67	<0.001	Yes
25 mg/mL vs Ciprofloxacin	15.77	<0.001	Yes
25 mg/mL vs Negative Control	8.30	<0.001	Yes
12.5 mg/mL vs 6.25 mg/mL	2.63	<0.001	Yes
12.5 mg/mL vs Ciprofloxacin	18.80	<0.001	Yes
12.5 mg/mL vs Negative Control	5.27	<0.001	Yes
6.25 mg/mL vs Ciprofloxacin	21.43	<0.001	Yes
6.25 mg/mL vs Negative Control	2.63	<0.001	Yes
Ciprofloxacin vs Negative Control	24.07	<0.001	Yes

Appendix E MIC determination

Table E1. Broth microdilution observations used for determination of MIC

Concentration (mg/mL)	**Observation**
100	No growth
50	No growth
25	No growth
12.5	Growth observed
6.25	Growth observed
3.125	Growth observed
1.56	Growth observed
0.78	Growth observed

Minimum Inhibitory Concentration (MIC) = 25 mg/mL

Appendix F Preparation of reagents

Preparation of 50% Ethanol

- 416.7 mL of 60% ethanol
- 83.3 mL distilled water

Preparation of 10% Sodium Hydroxide

- 20 g NaOH
- Distilled water to 200 mL

Preparation of 5% Ferric Chloride

- 5 g $FeCl_3 \cdot 6H_2O$
- Distilled water to 100 mL

Appendix G sample calculations

Mean Zone of Inhibition

For 100 mg/mL:

$$\text{Mean} = \frac{15.1 + 15.3 + 14.9}{3}$$

$$= 15.1 \text{ mg/mL}$$

Standard Deviation (SD)

SD calculation for 100mg/mL:

Data: 15.1, 15.3, 14.9

Mean ($\bar{x}$) = (15.1 + 15.3 + 14.9) / 3
Mean ($\bar{x}$) = 45.3 / 3
Mean ($\bar{x}$) = 15.1

Sample Standard Deviation (SD):

$$SD = \sqrt{[\Sigma(x - \bar{x})^2 / (n - 1)]}$$

$$= \sqrt{[(0^2 + 0.2^2 + (-0.2)^2) / (3 - 1)]}$$

$$= \sqrt{[(0 + 0.04 + 0.04) / 2]}$$

$$= \sqrt{(0.08 / 2)}$$

$$= \sqrt{0.04}$$

$$= 0.2$$

Result: 15.1 ± 0.2 mm (mean ± SD)