Combined Effects of Vitamin C & Food-Derived Ingredients Against Multidrug-Resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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A thesis submitted to the Program of Biotechnology in the Department of Mathematics and Natural Sciences (MNS) in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology

Department of Mathematics and Natural Sciences (MNS)

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Declaration

It is hereby declared that

1. The thesis submitted is our original work while completing the degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis has not been submitted or accepted for any other degree or diploma.
4. We have acknowledged all major sources of help.

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Abstract

The growing interest in food-derived antimicrobial therapy is driven by the expectation that natural compounds with antibacterial properties might serve as adjunctive strategies against antimicrobial resistance. This study examined whether common food-derived compounds can modify the activity of ciprofloxacin against multidrug-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*, both listed in the 2024 WHO Bacterial Priority Pathogens List. Vitamin C, turmeric, black seed, garlic, gallic acid and vanillin were prepared as 2 g/10 ml stocks and tested by disc diffusion at 5–15 µl per disc, giving total loads of roughly 300–3000 µg of natural compound. Ciprofloxacin (30 µg per disc) was used as the antibiotic control. On their own, the food-derived agents showed little or no inhibition at doses comparable to normal intake, and clear zones appeared only when vitamin C, gallic acid or vanillin were pushed into the upper hundreds of micrograms per disc. Ciprofloxacin was then combined with vitamin C plus either gallic acid or vanillin at stock ratios of 4:6, 3:7 and 2:0, keeping the ciprofloxacin dose at 30 µg while varying the total phenolic load between about 300 and 3000 µg per disc. In *S. aureus*, several gallic-acid–vitamin-C mixtures at 4:6 and 3:7 gave zones up to about 40 mm, compared with 37–38 mm for ciprofloxacin alone, indicating a modest synergistic effect. In contrast, vanillin-rich mixtures and many *P. aeruginosa* combinations produced smaller zones, around 25–30 mm where the ciprofloxacin control was 37–39 mm, consistent with antagonism. Some intermediate ratios were essentially indifferent. Overall, vitamin C and food-derived phenolics showed both synergistic and antagonistic interactions with ciprofloxacin, depending on dose and ratio, highlighting the need for careful optimisation before considering such combinations as antimicrobial adjuvants.Although the study is limited to an in vitro model, the results underscore the importance of further evaluating how antioxidant-rich supplements and dietary phenolics may enhance or complement antibiotic function. These observations support continued investigation in mammalian cell lines and in vivo systems to clarify their safety, pharmacokinetic behavior, and potential clinical benefits when used alongside conventional antibiotic therapy.

Dedication

This is dedicated to our family, our best friends, and all the amazing people in our lives who did not give up on us and motivated us through it all. Lastly, we dedicate this to ourselves for making it through the end, together with hard work, efforts, and love

Acknowledgment

We would like to start by sincerely thanking Almighty Allah for giving us the strength and endurance to finish our thesis. Without His favor and direction during this journey, this achievement would not have been feasible.

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List of Acronyms

AMR	Antimicrobial Resistance
GNB	Gram-Negative Bacteria
GPB	Gram-positive Bacteria
MIC	Minimum Inhibitory Concentration
MDR	Multi-Drug Resistant
MRSA	Methicillin-resistant *Staphylococcus aureus*
DMSO	Dimethyl sulfoxide
MHA	Mueller Hinton Agar
XLD	Xylose Lysine Deoxycholate
MIU	Motility Indole Urea
TSI	Triple Sugar Iron
MSA	Mannitol Salt Agar
NA	Nutrient Agar
AST	Antibiotic Susceptibility Test
CFU	Colony Forming Unit
FIC	Fractional Inhibitory Concentration

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Chapter 1: Introduction

1.1 Background

Antimicrobial resistance has been shown to complicate the treatment of chronic and recurrent infections and to place a substantial burden on clinical practice, while the introduction of new antibiotic classes has remained limited, so that the effectiveness of traditional antimicrobial therapies has continued to be relied upon within a narrowing therapeutic space. The main obstacle to developing and introducing novel antibiotic classes has been identified as the complex adaptive mechanisms that have evolved in bacteria, including reduced intracellular antibiotic accumulation through altered permeability and efflux, survival under oxidative stress, quorum-sensing-driven coordination of pathogenic biofilms, and the persistence of mature biofilms within the host despite antimicrobial penetration. In response to these constraints, the combination of established antibiotics with therapeutically weak or inactive adjuncts has been proposed as a pragmatic strategy, because such adjuncts can be used to modify antibiotic efficacy, sometimes improving it and sometimes reducing it, while doses remain within standard therapeutic ranges (Liu et al., 2024; Doern, 2014). Within this strategy, particular emphasis has been placed on plant-derived bioactive compounds that are economically accessible and generally recognized as non-toxic and biocompatible at oral exposure levels, since these agents can be integrated more readily into treatment concepts. These adjuncts have been described as interfering with several bacterial stress-response pathways and may either complement or counteract the action of conventional antimicrobials, so they are better viewed as modulators of antibiotic activity rather than guaranteed boosters

1.2 Rationale

Multidrug-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* are common hospital pathogens that are difficult to treat and are both listed in the WHO bacterial priority pathogens list. For many of these isolates, ciprofloxacin remains one of the few oral agents that still shows reliable in vitro activity. At the same time, patients frequently consume vitamin C and various plant-derived products, either as part of their normal diet or as over-the-counter supplements, during antibiotic treatment. These practices are often encouraged on the assumption that natural compounds will support immune function or help antibiotics work better, but they are rarely guided by microbiological evidence.

Although many studies have explored natural compounds as antibiotic adjuvants, there is still limited and sometimes conflicting evidence on how vitamin C and common phenolic food molecules affect ciprofloxacin activity against MDR *S. aureus* and *P. aeruginosa*. This thesis addresses that uncertainty by testing defined combinations under controlled in vitro conditions. The central question is not only whether these compounds can increase ciprofloxacin activity, but also whether they might leave it unchanged or even reduce it at the practically achievable doses. In this context, vitamin C, gallic acid, and vanillin were selected as model food-derived molecules because they are widely used, chemically well defined, and known for antioxidant and biofilm-modulating properties. A fixed 2:2:1 ratio of food molecule, vitamin C, and ciprofloxacin was applied in disc diffusion assays against MDR clinical isolates of *S. aureus* and *P. aeruginosa*, with the ciprofloxacin dose kept constant so that any change in zone size reflects the effect of the added compounds. Disc diffusion provides a simple phenotypic readout that can show both enhancement and loss of antibiotic activity, allowing increases in zone diameter to be read as possible synergy and decreases as antagonism or protection of the bacteria. Overall, the study aims to generate clear data on how these food-derived molecules and vitamin C modulate ciprofloxacin and to provide evidence that can guide safer use of dietary supplements alongside antibiotic therapy.

1.3 Significance of the Study

This study contributes to the growing field of antibiotic adjuvant therapy by providing experimental evidence that food-derived bioactive compounds can change the effectiveness of antibiotics against high-priority multidrug-resistant pathogens. The findings are presented as a scientific basis for deciding which combinations of antibiotics and common supplements should be encouraged, used with caution, or avoided, especially in low-resource settings. The observed interaction patterns, including instances where combinations reduced ciprofloxacin activity, can guide future work on efflux inhibition, biofilm disruption, and quorum interference by highlighting that helper compounds do not automatically restore antibiotic sensitivity and may sometimes blunt it. The potential significance of this work is particularly strong in settings where access to new and expensive antibiotics is limited and where multidrug-resistant infections are common. Food-derived compounds such as vitamin C, gallic acid, and vanillin are relatively

inexpensive, widely available, and familiar to the general population. If future work identifies conditions under which any of these molecules can safely enhance ciprofloxacin activity at realistic concentrations, they could form the basis of simple adjunct formulations, such as topical preparations or supplementary solutions, that do not require complex technology to produce. For example, a locally prepared cream or rinse containing safe levels of a phenolic compound that improves ciprofloxacin performance could provide an affordable addition to existing treatment protocols, especially in small hospitals and clinics.

1.4 Objectives

1. To systematically evaluate the standalone antibacterial activity and dose–response patterns of selected food-derived compounds (vitamin C, gallic acid, vanillin, turmeric and black seed) against multidrug-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

2. To investigate and classify the interaction profiles (synergistic, indifferent and antagonistic) between food-derived molecules, vitamin C and ciprofloxacin across defined concentration ranges using standardised disc diffusion assays.

3. To determine the specific concentration ratios at which phenolic compounds, particularly gallic acid and vanillin, either potentiate or attenuate ciprofloxacin activity, thereby identifying conditions that lead to meaningful enhancement or reduction of antibiotic efficacy.

Chapter 2: Literature Review

2.1 Global Context

Antimicrobial resistance was initially regarded primarily as a clinical concern, but it is now recognised to affect all areas of healthcare because antibiotics are becoming less effective precisely when they are required for procedures such as routine surgery, maternal and neonatal care, cancer treatment, and critical care. In May 2024, the World Health Organisation issued an updated Bacterial Priority Pathogens List that highlighted 24 antibiotic-resistant pathogens across 15 bacterial families worldwide and, for the first time, prompted widespread recognition of the need for targeted research, development, and stewardship focused on these organisms within health systems. This list, which now identifies antibiotic-resistant Gram-negative pathogens as a group requiring the most urgent study and clinical intervention, is expected to maintain and redirect attention toward other highly resilient, high-burden pathogens in both healthcare and community settings, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which are disproportionately responsible for severe bacterial disease (Sati et al., 2024). In this context, the systematic evaluation of safe and low-cost adjuvants that can alter antibiotic performance, for better or worse, is aligned with the World Health Organisation objective of prolonging antibiotic effectiveness in the face of a slow and uncertain antibiotic development pipeline, for example, when a non-antibiotic helper compound is used alongside a standard drug so that the same dose achieves better infection control (WHO, 2024). A widely cited economic review on antimicrobial resistance projected that, without effective action, drug-resistant infections could cause up to 10 million deaths per year globally by 2050, exceeding the mortality from many other major infectious diseases (O'Neill, 2016).

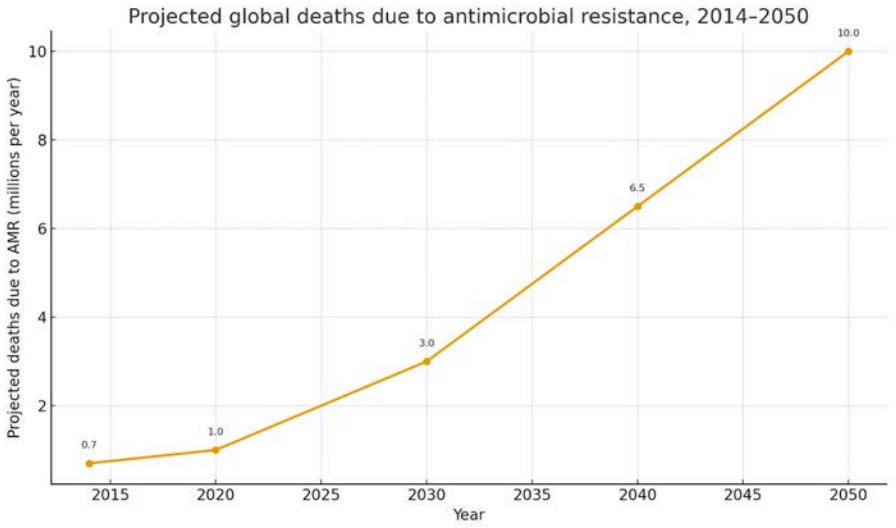

Figure 1. Projected global deaths due to antimicrobial resistance between 2014 and 2050, using commonly cited baseline estimates (~0.7 million deaths per year around 2014) and a worst-case projection of 10 million deaths per year by 2050. Intermediate values are shown for visualization of the rising trajectory.

2.2 Biological and Clinical Significance of the Target Organisms

The World Health Organisation has divided major antibiotic-resistant bacteria into critical, high, and medium priority tiers for research and development of new antibiotics. These tiers and the key resistance patterns are outlined in Table 1.

Table 1: WHO priority bacterial pathogens and key resistance patterns:

Priority tier	Pathogen	Key resistance pattern
PRIORITY 1: CRITICAL	*Acinetobacter baumannii*	Carbapenem-resistant
	Pseudomonas aeruginosa	Carbapenem-resistant
	Enterobacteriaceae	Carbapenem-resistant; third-generation cephalosporin-resistant
PRIORITY 2: HIGH	*Enterococcus faecium*	Vancomycin-resistant
	Staphylococcus aureus	Methicillin-resistant, vancomycin-intermediate, vancomycin-resistant
	Helicobacter pylori	Clarithromycin-resistant
	Campylobacter spp.	Fluoroquinolone-resistant
	Salmonella spp.	Fluoroquinolone-resistant
	Neisseria gonorrhoeae	Third-generation cephalosporin-resistant; fluoroquinolone-resistant
PRIORITY 3: MEDIUM	*Streptococcus pneumoniae*	Penicillin-nonsusceptible
	Haemophilus influenzae	Ampicillin-resistant
	Shigella spp.	Fluoroquinolone-resistant

(adapted from Tacconelli et al., WHO priority list for R&D of new antibiotics, 2017)

This thesis focuses on two pathogens from this list. *Pseudomonas aeruginosa* belongs to the critical priority tier as a carbapenem-resistant organism, and *Staphylococcus aureus* belongs to the high priority tier as a methicillin-resistant and sometimes vancomycin-resistant organism. Both are important causes of difficult-to-treat infections and therefore provide a relevant model system for testing potential adjuvant compounds.

Staphylococcus aureus is recognised as a clinically important pathogen because it causes severe diseases such as skin and soft tissue infections, osteomyelitis, endocarditis, pneumonia, and infections associated with medical devices, and it has become one of the most frequent examples of reduced fluoroquinolone susceptibility through combined molecular changes. In *S. aureus*, mutations in DNA gyrase and topoisomerase IV decrease fluoroquinolone binding, while overexpression of the NorA efflux pump actively exports drugs such as ciprofloxacin, so that intracellular antibiotic concentrations fall and the effective therapeutic window narrows, which facilitates rapid clinical resistance and allows treatment failure at standard doses (Palazzotti et al., 2023). These effects are reinforced by biofilm associated tolerance, because extracellular polymeric substances, local chemical gradients, and reduced drug activity create diffusion barriers and zones of low antibiotic exposure, so that fluoroquinolone efflux, target modification, and biofilm protection together raise the tolerance of the population; in this setting, even small shifts in intracellular ciprofloxacin concentration can cause large changes in measured susceptibility, which explains why low dose potentiation strategies can meaningfully alter *S. aureus* responses (Truong Bolduc et al., 2024; Liu et al., 2024).

Pseudomonas aeruginosa is recognised as having a different but equally layered defence architecture that includes low outer membrane permeability, multiple resistance nodulation division family efflux pumps, quorum-regulated virulence networks, and robust biofilm formation, which together produce a phenotype that appears intrinsically tolerant even before additional resistance determinants are acquired. In *P. aeruginosa*, quorum-sensing systems regulate the production of virulence factors and the maturation of biofilms, which in turn influence susceptibility to various antibiotics by enhancing community defences. Research suggests that strategies aimed at disrupting biofilms, interfering with quorum signalling, or decreasing efflux activity may enhance the efficacy of accompanying antibiotics. However, the

improvements tend to be modest and highly dependent on dosage due to the presence of multiple concurrent barriers (Elfadadny et al., 2024). As a result, disc diffusion combinations typically show smaller changes in inhibition zone size for *P. aeruginosa* than for *S. aureus*, a pattern that is consistent with experimental observations in which *S. aureus* generally shows more noticeable shifts in zone size, while *P. aeruginosa* tends to change more modestly and often in a strain- and compound-specific way.

2.3 Multi-drug Resistant *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium that is typically present on human skin and in the nasal passages as a harmless commensal, but it is also able to act as a significant opportunistic pathogen that causes severe infections such as bacteremia, pneumonia, osteomyelitis, and surgical site infections. Its ability to acquire and express multiple resistance determinants has resulted in its recognition as one of the most clinically important multidrug-resistant pathogens.

The emergence of methicillin-resistant *S. aureus* (MRSA) represented a major turning point in antimicrobial therapy, because these strains developed resistance not only to β-lactam antibiotics but also to other important classes, including macrolides, aminoglycosides, tetracyclines, and fluoroquinolones, through a combination of chromosomal mutations, mobile genetic elements, and active transport mechanisms that expel drugs from the cell. Resistance mechanisms in *S. aureus* include alterations of antibiotic target sites, enzymatic inactivation of drugs, and the activation of efflux pumps such as NorA, MepA, and TetK, which actively remove a wide range of antibiotics from the cytoplasm and thereby lower intracellular concentrations; NorA activity, in particular, has been associated with reduced susceptibility to fluoroquinolones such as ciprofloxacin. The organism also forms structured biofilms composed of cells embedded in a self-produced matrix of polysaccharides, proteins, and extracellular DNA, which shield bacteria from antibiotic penetration and host immune defenses, and biofilm-associated cells can be up to a thousand times more resistant to antimicrobial agents than planktonic cells. In addition, quorum-sensing systems such as the accessory gene regulator (agr) are used to coordinate virulence and resistance gene expression in response to environmental signals. These features target modification, drug inactivation, efflux, biofilm development, and regulated gene

expression, collectively contributing to *S. aureus*'s status as a persistent and formidable multidrug-resistant pathogen in clinical environments (Palazzotti et al., 2023; Truong Bolduc et al., 2024; Liu et al., 2024).

2.4 Multi-drug Resistant *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that is often found in severe infections in patients with weakened immunity, including those with burns, cystic fibrosis, or implanted medical devices, and it is recognized for strong natural resistance to many antibiotics as well as a capacity to gain new resistance through genetic changes and gene transfer. This resistance is explained by several layers of defence, including an outer membrane with very few pores that limit entry of water-loving drugs such as many β-lactams and fluoroquinolones; multiple resistance nodulation cell division family efflux pumps such as MexAB OprM, MexCD OprJ, and MexXY OprM that actively transport a wide range of unrelated antibiotics back out of the cell; and enzymes such as AmpC and extended-spectrum β-lactamases that chemically break β-lactam drugs, for example, when a β-lactam antibiotic is inactivated before it can reach its target. In addition, *P. aeruginosa* is known to form strong biofilms, which are structured layers of cells embedded in a matrix rich in polysaccharides such as alginate that act as physical and metabolic barriers, support slow-growing or dormant cells, and reduce the speed and depth with which antibiotics diffuse, for example, on the surface of a ventilator tube where a dense film slows drug penetration. These behaviors are further coordinated by quorum-sensing networks, including the Las, Rhl, and PQS systems, which regulate biofilm growth, virulence factor production, and adaptive responses to antibiotic exposure. Taken together, these traits create a model organism for multidrug resistance in which structural barriers, active efflux, drug-destroying enzymes, and regulated biofilms all work together to reduce antibiotic effectiveness, and for this reason *P. aeruginosa* has been placed on the World Health Organization Critical Priority Pathogen List, which has been used to justify the search for new strategies such as combining standard antibiotics with helper compounds to restore and improve treatment outcomes. (Elfadadny et al., 2024; WHO, 2024; Touati et al., 2025)

2.5 Ciprofloxacin

Ciprofloxacin is known as a broad-spectrum fluoroquinolone antibiotic that is widely used against many Gram-positive and Gram-negative bacteria. It acts by binding to and inhibiting the bacterial enzymes DNA gyrase and topoisomerase IV, which normally introduce and relax twists in DNA so that replication and transcription can proceed. So, when a stable drug-enzyme-DNA complex is formed, DNA strands are not resealed, and double-strand breaks accumulate. The bacterial cell is killed, as seen, for example, when a urinary tract infection is cleared because the causative *Escherichia coli* population can no longer replicate. (Doern, 2014; Shariati et al., 2022; Spencer et al., 2023). Over recent decades, increasing fluoroquinolone resistance has been observed, and several main mechanisms have been defined, including point mutations in the quinolone resistance determining regions of the gyrA and parC genes that change the shape of the drug binding site, reduced outer membrane permeability in Gram negative bacteria that slows entry, overexpression of efflux pumps such as NorA in *Staphylococcus aureus* and MexAB OprM in *Pseudomonas aeruginosa* that actively export ciprofloxacin out of the cell, and plasmid mediated quinolone resistance genes such as qnr, which protect topoisomerases, and aac(6′) Ib cr, which chemically modifies ciprofloxacin, for example when a clinical isolate with gyrA and parC mutations plus a qnr plasmid requires much higher ciprofloxacin concentrations to inhibit growth. (Palazzotti et al., 2023; Truong Bolduc et al., 2024; Elfadadny et al., 2024; Jiang et al., 2016) Because these layered mechanisms reduce intracellular drug levels and weaken killing, the restoration of ciprofloxacin activity through adjuvant therapy is presented as a rational strategy. in which ciprofloxacin is combined with safe non-antibiotic compounds that alter efflux activity, membrane permeability, or biofilm stability so that more drug reaches its targets without increasing the antibiotic dose, as would be expected, for example, if gallic acid partially inhibits efflux and vitamin C weakens biofilm matrices, leading to larger inhibition zones at the same ciprofloxacin load in a diffusion assay. (Doern, 2014; Touati et al., 2025; Shariati et al., 2022)

2.6 Vitamin C

Vitamin C is an antioxidant that dissolves in water and has many important jobs in the body. It is becoming more well-known for its ability to fight germs and biofilms. Its adjuvant potential primarily arises from redox modulation that interferes with bacterial oxidative stress regulation. In vitro, vitamin C can participate in redox cycling, which increases the levels of reactive oxygen species. It weakens the extracellular polymeric substance matrix in biofilms and makes it harder for things to stick at first to surfaces, and it makes the membrane more permeable so that partner antibiotics can get into cells more easily. Vitamin C is not a strong bactericide on its own at low, non-toxic levels. It can make different types of bacteria more sensitive to regular antibiotics by changing the way they work inside cells. Situations that cause stress, which makes antibiotics work better. Practical factors include chemical lability and oxidation in aqueous environments, which necessitate fresh preparation and consistent handling in diffusion or MIC assays. In the context of combination therapy, vitamin C is often described as a secure, economical modulator of redox balance and biofilm structure. At the same time, several reports show that high levels of vitamin C and other antioxidants can protect bacteria from fluoroquinolone killing, so its overall effect on antibiotic activity appears to be context-dependent. (Liu et al., 2024; Doern, 2014).

2.7 Adjuvants Screened

Two phenolic adjuvants, gallic acid and vanillin, were prioritized on mechanistic grounds along with vitamin C, while turmeric (Curcuma longa) and black seed (Nigella sativa) were retained as comparators. Gallic acid was selected because it acts on bacterial membranes, chelates metal ions that enzymes require, and may modulate efflux systems so that intracellular levels of partner antibiotics such as ciprofloxacin can be increased at the same external dose; in simple terms, earlier studies have reported slightly larger clear zones around antibiotic discs supplemented with gallic acid compared with antibiotic alone, which encouraged us to include it in our panel. Vanillin was chosen because it interferes with quorum sensing and biofilm formation, which are community-level defenses that reduce antibiotic effectiveness without always changing minimum inhibitory concentrations in free-floating culture, so disruption of these signals can permit better antibiotic penetration and reduce adaptive resistance behaviors. In the first screening phase, turmeric, black seed, ginger, and garlic were tested individually at practical

exposure levels against the target organisms, but they did not produce measurable zones of inhibition in disc diffusion assays, whereas subsequent tests with gallic acid and vanillin produced clearer and more consistent halos under the same basic conditions. On this basis, turmeric, black seed, ginger, and garlic were treated as low-activity or negative controls and considered as possible background adjuncts rather than primary bacteriostatic agents, while gallic acid and vanillin were advanced into fixed-ratio combination experiments designed to measure changes in ciprofloxacin activity (Liu et al., 2024; Palazzotti et al., 2023; Touati et al., 2025).

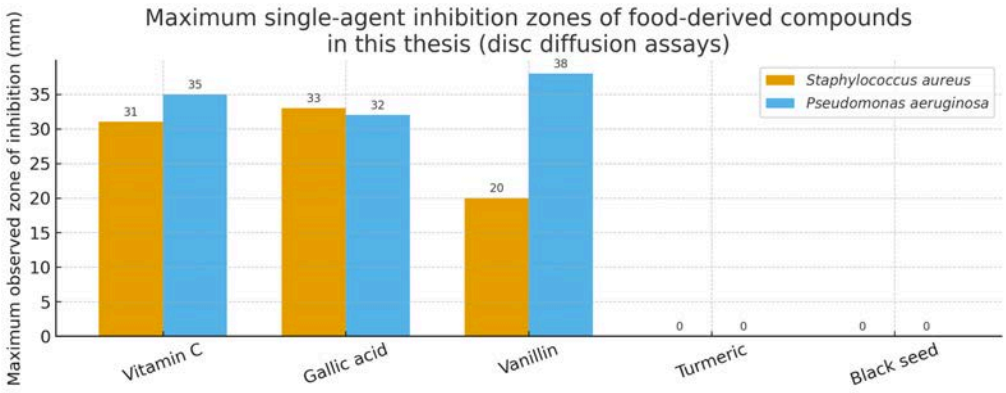

Figure 2. Maximum single-agent zones of inhibition for the food-derived compounds used in this study, measured by disc diffusion against *Staphylococcus aureu*s and *Pseudomonas aeruginosa.* Turmeric and Black seed showed no measurable activity, whereas Vitamin C, Gallic acid, and Vanillin produced inhibition zones in at least one of the test organisms

2.8 Gallic Acid

Gallic acid is a trihydroxybenzoic acid and a phenolic compound that is associated with antioxidant, anti-inflammatory, and antimicrobial properties, and its antibacterial effects are understood to arise from several complementary mechanisms that include disruption of cytoplasmic and outer membranes, chelation of metal ions required by bacterial enzymes, inhibition of key metabolic enzymes, and interference with transport systems. A particular emphasis is placed on its reported ability to reduce the effective activity of efflux pumps in resistant bacteria so that active extrusion of partner antibiotics is limited and higher intracellular concentrations are achieved at the same external dose. In this way, restoration or enhancement of

the activity of antibiotics such as ciprofloxacin can be observed without increasing antibiotic mass, for example, when a larger and clearer zone of inhibition is measured around a ciprofloxacin disc that has been combined with a low microgram amount of gallic acid compared with a disc containing ciprofloxacin alone. In biofilm settings, gallic acid is described as compromising matrix integrity and weakening collective defenses, which permits deeper antibiotic penetration into the biofilm structure and exposes a larger fraction of the bacterial population to the drug. Taken together, these characteristics have been used to justify gallic acid as a phenolic component in fixed-ratio combinations. However, reported effects range from potentiation to antagonism, so its impact on antibiotic action has to be determined experimentally in each setting (Palazzotti et al., 2023; Doern, 2014)

2.9 Vanillin

Vanillin is described as a naturally occurring phenolic aldehyde that is derived from lignin and that possesses antioxidant, antimicrobial, quorum quenching, and antibiofilm properties. Its antimicrobial role is presented as weakening collective bacterial defenses rather than acting as a strong direct killer at practical exposure levels. Vanillin is reported to interfere with quorum-sensing pathways, which leads to slower biofilm maturation and reduced coordination of virulence factor production, and it is also associated with mild disturbance of cell membranes and modest inhibition of selected enzymes, so that a local microenvironment is created in which antibiotics encounter fewer community-level barriers. In this setting, vanillin has been proposed to enhance antibiotic performance by limiting quorum signalling and biofilm growth, although some studies also report neutral or opposing effects. This underlines the need to test vanillin–antibiotic combinations rather than assuming they will always lower the effective dose needed for growth inhibition. Because vanillin is generally regarded as biocompatible and cost-effective, these combined features are used to justify its selection as a phenolic co-adjuvant in translational strategies that pair conventional antibiotics, and optionally vitamin C, with mechanism-complementary helper compounds to target multidrug-resistant pathogens. (Touati et al., 2025; Doern, 2014).

2.10 Identified Research Gaps

The literature reviewed in this chapter has shown that *Staphylococcus aureus* and *Pseudomonas aeruginosa* possess multiple and layered mechanisms of resistance, including biofilm formation, altered targets, efflux pumps, and reduced permeability. Ciprofloxacin has been widely used against these organisms, but its activity is increasingly compromised by these defenses. A range of plant- and food-derived compounds, including vitamin C, gallic acid, and vanillin, has been reported to display antimicrobial, antibiofilm, or adjuvant properties through different biochemical pathways. These findings suggest that such molecules can act as helper agents that modify antibiotic activity, but they may either restore, leave unchanged, or reduce the effect of the partner drug.

At the same time, several important gaps in the literature have become apparent. Most studies have focused on single compounds and have not compared vitamin C, gallic acid, and vanillin directly as adjuvants for ciprofloxacin under one unified design. Many reports have used complex or highly specific assays, such as advanced MIC or time kill setups, which are not always accessible in routine microbiology laboratories. In addition, relatively few experiments have examined these compounds against *S. aureus* and *P. aeruginosa* together, using standardised conditions that allow clear comparison of responses between the two species. In simple terms, the literature often explains how these compounds may work in theory, but it rarely shows which of them helps ciprofloxacin the most when they are tested in the same way. To address these gaps, the present study tests fixed-ratio combinations of ciprofloxacin with vitamin C, gallic acid, and vanillin against MDR clinical isolates, focusing on both possible enhancement and reduction of ciprofloxacin activity. This identified gap provides the basis and direction for the experimental work presented in the following chapters.

Chapter 3: Materials and Methods

3.1 Methodology and Workflow:

Clinical isolates were taken from the IFST(Institute of Food Science and Technology), Microbiology Lab of BCSIR Dhaka. The isolates were then cultured in nutrient agar plates and later subcultured on selective media like cetrimide agar and mannitol salt agar to guarantee purity and viability of *P. aeruginosa* and *S. aureus*.Bacterial stock preparation was done, and the isolates were stocked in T1N1 agar. To accurately identify the strains, a few biochemical tests were run, Gram staining , the Citrate utilization test , Motility, Indole, Urease (MIU), and the triple sugar iron (TSI) test. Molecular identification was done too by extracting the DNA of the isolates, followed by PCR amplification for confirming the strains. Antibiotic Susceptibility Testing (AST) was used to assess how well various antibiotics work against the bacterial isolates used in this study and to further understand if the strains are multi-drug resistant. The antibiotic ciprofloxacin was selected, and the Minimum Inhibitory Concentration (MIC) was performed for Vitamin C, gallic acid, and ciprofloxacin, keeping standard positive and negative controls. Synergy testing with various concentrations between selected agents and combination treatments was done, and zones of inhibition were measured after incubation to evaluate and compare antibacterial effectiveness

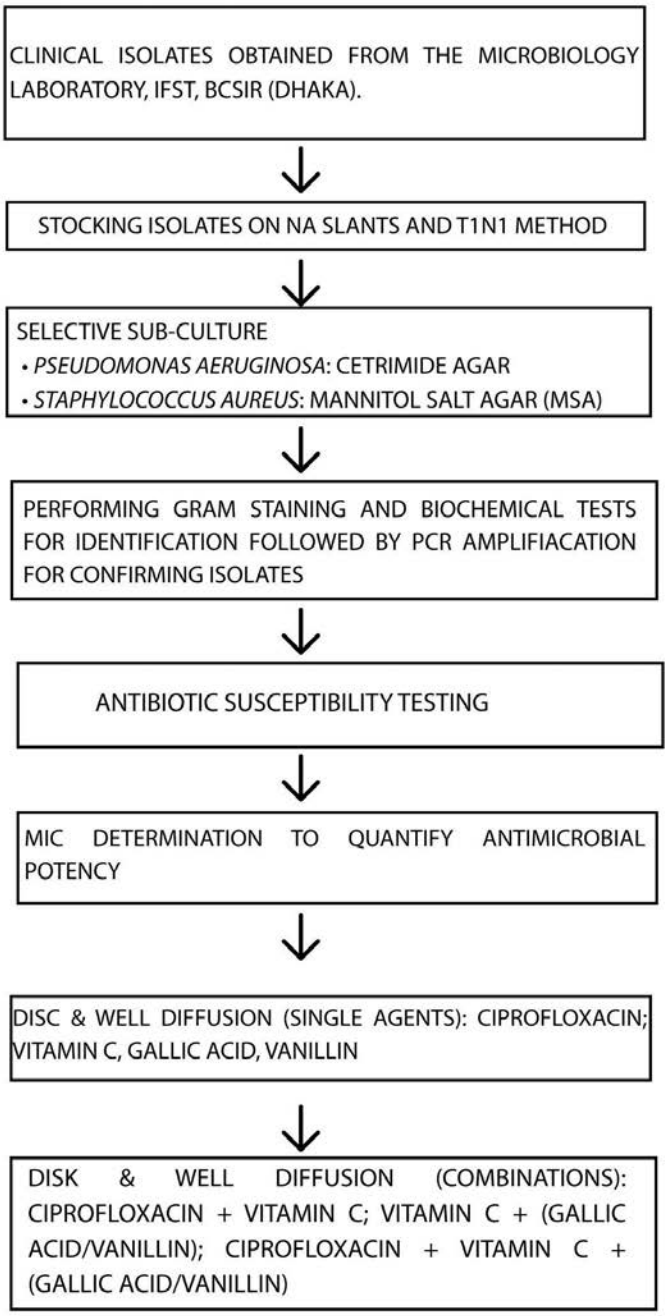

Figure 3: General workflow of the entire experiment

3.2 Workplace for the Study:

This experiment was completed at the Communicable Disease and Biomarkers (CDAB) Laboratory of the Department of Mathematics and Natural Sciences , BRAC university.

3.3.Sample collection site and processing:

Clinical isolates of *Pseudomonas* and *Staphylococcus* from gut associated samples were obtained from the IFST Microbiology Laboratory, BCSIR (Dhaka). The isolates were preserved in glycerol stocks and were transported to BRAC University using an insulated icebox to ensure cellular viability and to ensure optimal temperature conditions during the transfer process.

The isolates were revived from the glycerol stock and cultured in Nutrient Agar (NA) and was incubated at 37°C for 24 hours; after that, the visible colonies were observed. Using the streak plate method, the culture were subcultured again in Nutrient Agar (NA) and incubated for 24 hours at 37°C to ensure active growth and optimum colony development

After visible growth were seen , species specific subculture were done *Pseudomonas aeruginosa* was subcultured on Cetrimide Agar, and *Staphylococcus aureus* was subcultured on Mannitol Salt Agar (MSA). For later use and long term storage T1N1 agar slants were used for each strains , vials were sealed with parafilm to maintain viability.

3.4 Preparation of Media:

Various types of media were used throughout the study

Table 02: types of media used in the experiment

Media	Purpose
Nutrient Agar (NA)	It was used for bacterial cultivation and subculture purposes
Mannitol Salt Agar (MSA)	It was used to isolate and identify Staphylococcus aureus
Cetrimide Agar	It was used for the selective isolation and presumptive identification of *Pseudomonas aeruginosa*.
Mueller Hinton Agar (MHA)	It was used for testing for antibiotic susceptibility
T1N1 agar	It is used for bacterial stock preparation

3.4.1. Nutrient Agar (NA) Media

- The preparation of nutrient agar was carried out by measuring 28.0 g of the medium for every 1000 ml of distilled water.
- This ratio was used as the base formula for later media preparation
- The mixture was heated and stirred until the powder dissolved uniformly.
- The prepared solution was then sterilized in an autoclave at 121°C for fifteen minutes.
- After sterilization, the medium was allowed to cool, and it was poured into sterile Petri dishes

3.4.2 Mueller Hinton Agar (MHA) Media

- The preparation of MHA was carried out by measuring 38.0 g of the medium for every 1000 ml of distilled water.
- This ratio was used as the base formula for later media preparation

- The mixture was heated and stirred until the powder dissolved uniformly.
- The prepared solution was then sterilized in an autoclave at 121°C for fifteen minutes.
- After sterilization, the medium was allowed to cool, and it was poured into sterile Petri dishes

3.4.3 Cetrimide Agar

- The preparation of Cetrimide Agar was carried out by measuring 45.3 g of the medium for every 1000 ml of distilled water.
- This ratio was used as the base formula for later media preparation
- The mixture was heated and stirred until the powder dissolved uniformly.
- The prepared solution was then sterilized in an autoclave at 121°C for fifteen minutes.
- After sterilization, the medium was allowed to cool, and it was poured into sterile Petri dishes

3.4.4 Xylose Lysine Deoxycholate (XLD)

- The preparation of XLD Agar was carried out by measuring 52.0 g of the medium for every 1000 ml of distilled water.
- This ratio was used as the base formula for later media preparation
- .The mixture was heated and stirred until the powder dissolved uniformly.
- The prepared solution was then sterilized in an autoclave at 121°C for fifteen minutes.
- After sterilization, the medium was allowed to cool, and it was poured into sterile Petri dishes

3.4.5 MSA (Mannitol Salt Agar) Media

- The preparation of MSA was carried out by measuring 110 g of the medium for every 1000 ml of distilled water.
- This ratio was used as the base formula for later media preparation
- The mixture was heated and stirred until the powder dissolved uniformly.

- The prepared solution was then sterilized in an autoclave at 121°C for fifteen minutes.
- After sterilization, the medium was allowed to cool, and it was poured into sterile Petri dishes

3.4.6 T1N1 Agar (Tryptone Salt Agar)

- For 1000 ml of T1N1 agar, 10 gm of tryptone, 10 gm of NaCl, and 20 gm of agar were needed to add to distilled water.
- The ingredients were suspended and boiled to dissolve properly.
- Two milliliters of T1N1 agar were poured into each three-milliliter sterile vial.
- The vials containing T1N1 agar were autoclaved at 121°C for 15 minutes.

3.5 Preparation of Bacterial Suspension

Physiological saline was used to create the bacterial suspension. Using a sterile loop, a small amount of bacteria was extracted from a single colony of a young culture and dissolved in saline. The solution was vortexed to guarantee that the bacteria were completely dissolved. For additional analysis, it was compared with solutions using the McFarland standard 0.5.

3.6 Subculture

Freshly made NA plates were utilized for the clinical isolates' subculture. Using a sterile loop, a single colony was selected and spread throughout the media using the streak plate technique.
To obtain pure cultures, the plates were then incubated for 24 hours at 37°C.

3.7 Preparation of Bacterial Stock Preparation

T1N1 agar was utilized to preserve the clinical isolates over time. Using a sterile inoculating needle, bacteria were injected into the agar medium three times. The vials were then incubated at 37°C for the entire night. Following incubation, 150 µl of sterile paraffin oil was applied to the medium's surface and kept at room temperature with proper labels

3.8 Preparation of food ingredient solution

Two grams of each food molecule powder were precisely weighed and dissolved in 10 ml of autoclaved distilled water. Dimethyl sulfoxide (DMSO) was added in small amounts for the solutions that were difficult to dissolve and thoroughly stirred to guarantee full dissolution. Sterile syringe filters were used to remove any undissolved particles, and the clear filtrates were collected aseptically in autoclaved beakers to create pure, homogenous solutions that could be used in experiments.

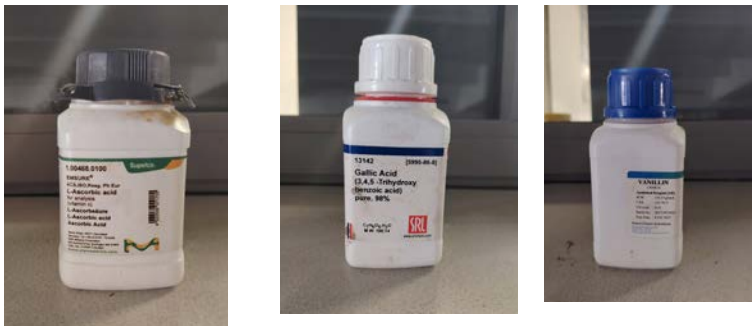

Figure 4: Ascorbic Acid (Vitamin C), Gallic Acid and Vanillin powder used in the experiment

3.9 Antibiotic Susceptibility Test (AST)

To find out the isolates' susceptibility to antibiotics, this test was carried out. For this, the disc diffusion technique was used. First, sterile cotton swabs were used to equally distribute bacterial solutions across Mueller Hinton Agar (MHA) plates. Two MHA plates were used for each strain, and each plate was given three antibiotic disks, amoxicillin, ciprofloxacin, ceftriaxone, azithromycin, kanamycin, and oxacillin. Thereafter, the plates were kept in the incubator for a full day at 37°C. The clear zones surrounding each antibiotic disk were measured and compared to established norms following the 24-hour incubation period. This comparison made it possible to identify which samples were responsive to particular medications and which antibiotics exhibited resistance

Table 03: Interpretation of Antibiotic Susceptibility for *Staphyloccus aureus* & *Pseudomonas aeruginosa*

Antibiotic Name	Antibiotic Group	Disk Conc	Symbol (mcg/disc)	*S. aureus* Result	P. aeruginosa Result
Amoxicillin	β-lactam antibiotic	10	AML	Resistant	Resistant
Ciprofloxacin	Fluoroquinoloe	5	CIP	Susceptible	Susceptible
Ceftriaxone	β-lactam antibiotic	30	CRO	Resistant	Susceptible
Azithromycin	Macrolide antibiotic	15	AZM	Resistant	Resistant
Kanamycin	Aminoglycoside antibiotic	30	K	Resistant	Resistant
Oxacillin	β-lactam antibiotic	1	OX	Resistant	N/A

3.10 Preparation of Physiological Saline

Biological suspensions of the bacteria were prepared using physiological saline. To achieve the required concentration of 0.9% sodium chloride, it was dissolved in 100 ml of distilled water. Subsequently, 10 ml of the saline solution was added to each test tube. The test tubes were then autoclaved at 121°C for 15 minutes and left at room temperature afterward.

3.11 Dilution ratio

For the dilution process, a stock solution of the test compound was made at a 2 g/10 mL concentration. Working dilutions were prepared from this stock using sterile distilled water in a manner that achieved progressively lower concentrations suitable for antimicrobial testing. The following sample-to-diluent ratios were used to prepare dilutions: 1:9, 2:8, 3:7, and 4:6, with thorough mixing at each step.

3.12 Biochemical Tests

3.12.1 Gram Staining

The inoculation loop was sterilized over the flame after a clean, grease-free glass slide was taken and appropriately labeled. Using a loopful of the sample, a smear of the suspension was created on the slide. Before staining, the slide was air-dried, heat-fixed by passing it over the spirit lamp, and then allowed to cool. After that, the slide was covered in crystal violet and left for a minute. The slide was held at a 45° angle while being gently cleaned with tap water. Gram iodine mordant was applied to the smear for one minute. Again, the slide was gently washed with tap water, holding it at 45°. Decolorized for 5-15 seconds with 95% ethanol. To avoid over-decolorization, drop-by-drop reagent was added until crystal violet did not wash away from the smear. Gently the slide was washed with tap water while holding it at 45. A counterstain with safranin was added and kept for 1 minute. Then the slide was washed with tap water while holding it at 45°. Allowed to dry before putting on the cover slip. Lastly, it was examined under a microscope.

3.12.2 Motility Indole Urea (MIU) test

Three tests in one tube aid in differentiating the organisms based on their motility, synthesis of urease, and indole. MIU agar was made and poured into test tubes, followed by autoclaving them. With a sterilized inoculating needle, a few colonies were stabbed directly in the agar to inoculate it. At 37°C, the cultures were incubated for 24 hours. Following that, medium color changes were checked to see whether growth had occurred.

3.12.3 Triple Sugar Iron (TSI) Test

After the TSI media was made it was autoclaved; media was poured in test tubes and slant-dried. After that, the slants were streaked and stabbed to inoculate the test tubes with a 24-hour culture. To ascertain the bacerial growth, their color change was monitored after a 24-hour incubation period at 37°C.

3.12.4 Citrate Utilization Test

Simmon's Citrate Agar was prepared, autoclaved, and poured into vials in the shape of slants. After that, these slants were streaked and stabbed with a culture that had been incubated for 18 to 24 hours. Bacterial growth was assessed by monitoring any color changes after the vials were incubated for 24 hours at 37°C.

3.13 Determination of Minimum Inhibitory Concentration (MIC)

A standardized bacterial inoculum, adjusted to the 0.5 McFarland turbidity standard to ensure reproducible starting density, was added to each well of a 96-well microtiter plate in the broth microdilution format. Growth is evaluated visually (turbidity/film) following an overnight incubation period. The antimicrobials were serially diluted (usually two-fold) across the plate.

After subculturing the test organism used to produce a fresh, actively growing suspension, sterile saline/broth and a turbidity standard were adjust to the 0.5 McFarland standard (≈1–2 × 10^1 CFU/mL). In order to improve comparability across plates and compounds, the adjusted suspension was further diluted in broth to produce the recommended working inoculum for microdilution, which targets ~5 × 10^3 CFU/mL as the final concentration in each well after inoculation.

Sterile, flat-bottom 96-well microtiter plates were used. For each test arm, two-fold serial dilutions of the prepared agents were made across the row/columns in cation-adjusted broth (or the appropriate medium for the organism). Three test series were set up: A (Antibiotic alone), B (Vitamin C), and C (Gallic acid). In parallel, two control series were included on the same plate: a Positive control consisting of wells with broth plus the standardized bacterial inoculum but no antimicrobial (verifies organism viability and expected turbidity), and a Negative control consisting of wells with broth and the test antimicrobial without bacteria (verifies reagent sterility and detects any intrinsic color/turbidity of compounds).

3.14 Molecular characterization

Molecular characterization is such a technique to identify and classify isolates that implies other techniques like DNA sequencing and polymerase chain reaction (PCR) by scrutinizing their genetic material. It makes us understand the genetic composition and components of bacteria by providing powerful techniques with a wide range of applications in research settings.

3.14.1 Bacterial Genomic DNA Extraction

For DNA extraction, a loopful of bacterial colony was transferred into 1 mL of TE buffer in a sterile microcentrifuge tube and thoroughly mixed to obtain a uniform suspension. A dry heat block was used for the mixture to be heated at 95°C for 10 minutes,in order to lyse the bacterial cells and release the DNA into the solution. The tube was immediately heated and then kept at -20°C for three minutes to promote the precipitation of cell debris. Following a high-speed centrifugation of the sample, the extracted DNA-containing supernatant was carefully collected for additional molecular analysis.

3.14.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was done for the identification and confirmation of *Staphylococcus aureus* and *Pseudomonas aeruginosa* using species-specific primers. Each reaction mixture had a total volume of 13 µL, consisting of 6.5 µL of Master Mix, 0.45 µL of Forward Primer (FP), 0.45 µL of Reverse Primer (RP), 3.6 µL of Nuclease-Free Water (NFW), and 2 µL of template DNA. The mixture containing all the reagents was mixed and centrifuged for a brief period before placing it into the thermal cycler.

For *Staphylococcus aureus*, amplification used NUC (F) and NUC (R) primers, producing a 279 bp fragment of the *nuc* gene. The PCR conditions were initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 90 s, with a final extension step at 72 °C for 10 min.

For *Pseudomonas aeruginosa*, amplification targeted the 16S rDNA gene using two separate primer sets - PA SS (F) ,PA SS (R), PA GS (F), and PA GS (R) primers—with an expected amplicon size of 956 bp. The thermocycling program included an initial denaturation at 95 °C for

2 min, followed by 25 cycles of denaturation at 94 °C for 20 s, annealing at 58 °C for 20 s, and extension at 72 °C for 40 s, finishing with a final extension at 72 °C for 1 min.

To avoid contamination, aseptic conditions and nuclease-free consumables were used for all reactions. The amplified products were kept at -20 °C until they were put through agarose gel electrophoresis to verify the purity and size of the bands.

Table 4: Selected primers for PCR amplification

Primer	**Nucleotide Sequence**	**Organism**	**Temperature (°C)**	**Product Size (bp)**
NUC	F-5'- GCG ATT GAT GGT GAT ACG GTT-3′ R-5'AGC CAA GCC TTG ACG AAC TAA AGC-3′	*Staphylococcus aureus* (nuc gene)	58	279
PA SS	F-5′-GGGGGATCTTCG GACCTCA-3′ R-5′-TCCTTAGAGTGC CCACCCG-3′	*Pseudomonas aeruginosa* (16S rDNA)	58	956
PA GS	F-5′-GACGGGTGAGTA ATGCCTA-3′ R-5′-CACTGGTGTTCC TTCCTATA-3′	*Pseudomonas* species (16S rDNA)	54	956

3.14.3 Gel Electrophoresis

Gel electrophoresis was performed in this experiment to verify the presence of PCR products at the desired location. A 1% agarose gel was prepared by mixing 1g of agarose with 100 ml of TAE buffer. The mixture was heated, thoroughly mixed, and then cooled to a semi-warm temperature. To this gel solution, 3 µl of EtBr was added and mixed. The casting tray was arranged with combs to accommodate multiple sample.The gel solution was poured into the

casting tray and allowed to solidify. Once solidified, the combs were gently removed, and the tray was placed into the gel electrophoresis machine. Then, TAE running buffer was added, and 6.5 µl of the 50 bp Ladder was carefully placed in the first well of each row, while 6.5 µl of the PCR products were loaded into the other wells. The gel was then run at 110 volts for 40 minutes. After the completion of gel electrophoresis, gel bands were observed using UV light, confirming the presence of PCR products at the intended site

Chapter 4.0: Results

The results section presents the biochemical, molecular, and antimicrobial assays performed to characterize the bacterial isolates and evaluate their responses to different antibiotics and treatment conditions. The study focused on two clinically significant species, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, both known for their adaptability and strong capacity to develop antimicrobial resistance. Biochemical assays and polymerase chain reaction (PCR) amplification were employed to verify the identity of the isolates. PCR served as a crucial component for confirming the genetic identity of the organisms, ensuring that the cultured samples corresponded accurately to *P. aeruginosa* and *S. aureus*. Biochemical characterization further supported this identification by assessing phenotypic features such as motility, urease activity, indole production, and citrate utilization, along with additional diagnostic markers.

In addition to these standard identification procedures, antibiotic susceptibility testing (AST) was performed to determine the resistance patterns of the isolates. PCR-based molecular confirmation strengthened the reliability of these findings and validated the isolates prior to antimicrobial evaluation. The results also include minimum inhibitory concentration (MIC) testing and both agar disc and well diffusion assays to assess bacterial responses to food-derived molecules, vitamin C, and selected antibiotics. Visual documentation of the AST plates, including zones of inhibition, illustrates the inhibitory patterns across different antibiotics and dosages. Furthermore, the MIC data, inhibition zones, and graphical summaries demonstrate how varying concentrations and combinations of vitamin C, food molecules, and antibiotics influenced bacterial growth. The experiments also explored potential synergistic or antagonistic interactions arising from these combinations. By comparing inhibition zones and MIC values across treatments, the results provide insight into whether combined therapies offer improved efficacy compared to antibiotics used alone.

4.1 Biochemical Tests

Identifying microorganisms holds substantial importance within microbiology and the broader biological sciences, as many bacterial species exert significant effects on human and animal health. Numerous unidentified strains are capable of producing valuable metabolites used in fields such as medicine, food processing, cosmetics, and public health. Accurate identification of these organisms enables researchers to recognize and utilize their beneficial properties. Conversely, unidentified pathogenic strains may contribute to diseases that threaten human and animal populations. Their identification allows medical practitioners and researchers to develop appropriate treatment strategies and implement effective control measures (Hafezi & Khamar, 2024). Biochemical profiling plays a key role in this process because it supports taxonomic classification and provides the foundational information required for further physiological, molecular, and antimicrobial testing.Their role involves assisting in the selection of confirmatory molecular assays, aiding in the understanding of trends in antimicrobial susceptibility, and offering context for phenotypes such as motility or urease production that are linked to virulence or environmental adaptation.

4.1.1 Biochemical Test Observation

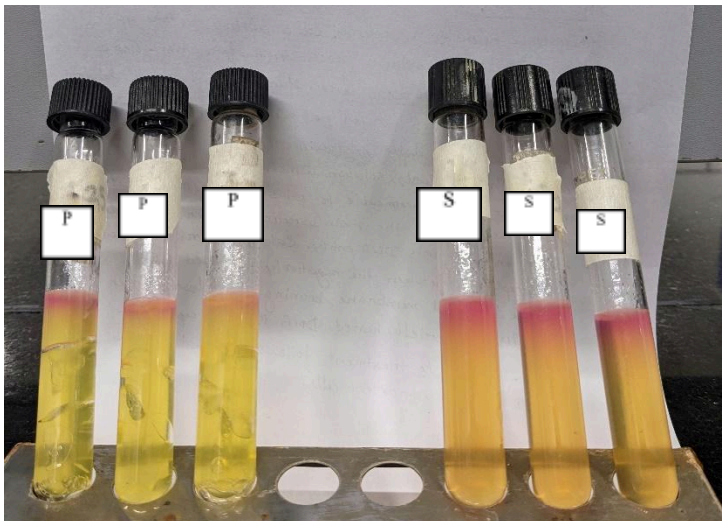

Fig. 5: Motility, Indole, and Urease Test.

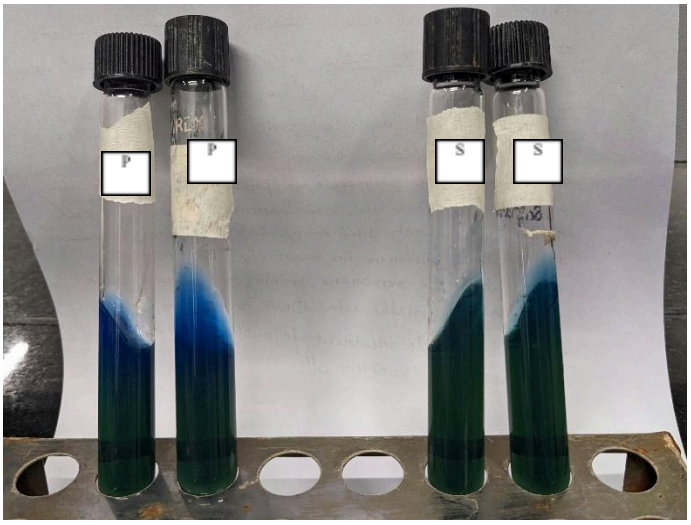

Fig. 6: Citrate Test.

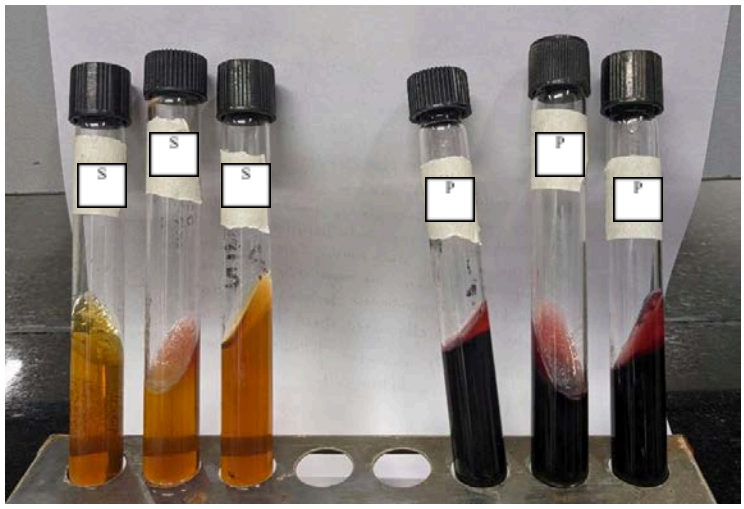

Fig. 7: Triple Sugar Iron Test.

S: *Staphylococcus aureus*

P: *Pseudomonas aeruginosa*

4.1.2 Table 05: Biochemical Test Results

Test	**Indicator**	***Pseudomonas aeruginosa***	***Staphylococcus aureus***
MIU – Motility	Cloudy growth	+	–
MIU – Indole	Red ring	–	–
MIU – Urease	Pink color	-	+
TSI – Slant/Butt	Red/yellow color	K/K (red/red)	A/A (yellow/yellow)
TSI – Gas	Cracks/bubbles	–	–
TSI – H_2S	Black ppt	–	–
Citrate	Blue/green color	+ (blue)	– (green)

4.1.3 Result Interpretation

- **MIU Test:** *Pseudomonas aeruginosa* explained the positive motility in the MIU test, yet the *Staphylococcus aureus* made an exhibition that is non-motile. Indole was never produced by the isolate, and it was proven by the absence of a red ring with the addition of Kovacs reagent. Even the test of urease has revealed that *P. aeruginosa* had been negative. *S. aureus* was tested as positive since the medium had a pink but distinct color.

- **TSI Test:** The triple sugar iron agar test has indicated that *P. aeruginosa* exhibited a red slope and a red butt (K/K). There were no splits or bubbles in the gas. Also there was no black precipitate (H_2S) that was created. Although S. *aureus* had delivered a yellow slant

and a yellow butt (A/A). It clearly indicates the acid production in the tube of both slant and butt. Furthermore, there was no creation of gas or H_2O that happened there.

- **Citrate Utilization Test:** The test of citrate had explained that *Pseudomonas aeruginosa* delivered a blue hue that indicates the capability to use citrate. *Staphylococcus aureus* managed to maintain the inherent green hue of the medium.

4.2 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) is a fundamental technique in molecular biology that enables the amplification of specific DNA sequences, allowing for highly sensitive and precise bacterial detection and identification. The method relies on short, single-stranded DNA fragments known as primers, which are carefully designed to bind to conserved regions within the genome of the target organism. During PCR, a thermal cycler repeatedly heats and cools the reaction mixture, resulting in denaturation of the double-stranded DNA, primer annealing, and extension by a thermostable DNA polymerase, ultimately generating millions of copies of the target sequence. Following amplification, gel electrophoresis is used to separate the PCR products based on fragment size, providing visual confirmation of successful amplification. The appearance of a clear, distinct band at the expected size, as compared with a molecular ladder, indicates the presence of the target organism in the sample.

4.2.1 Observation of PCR

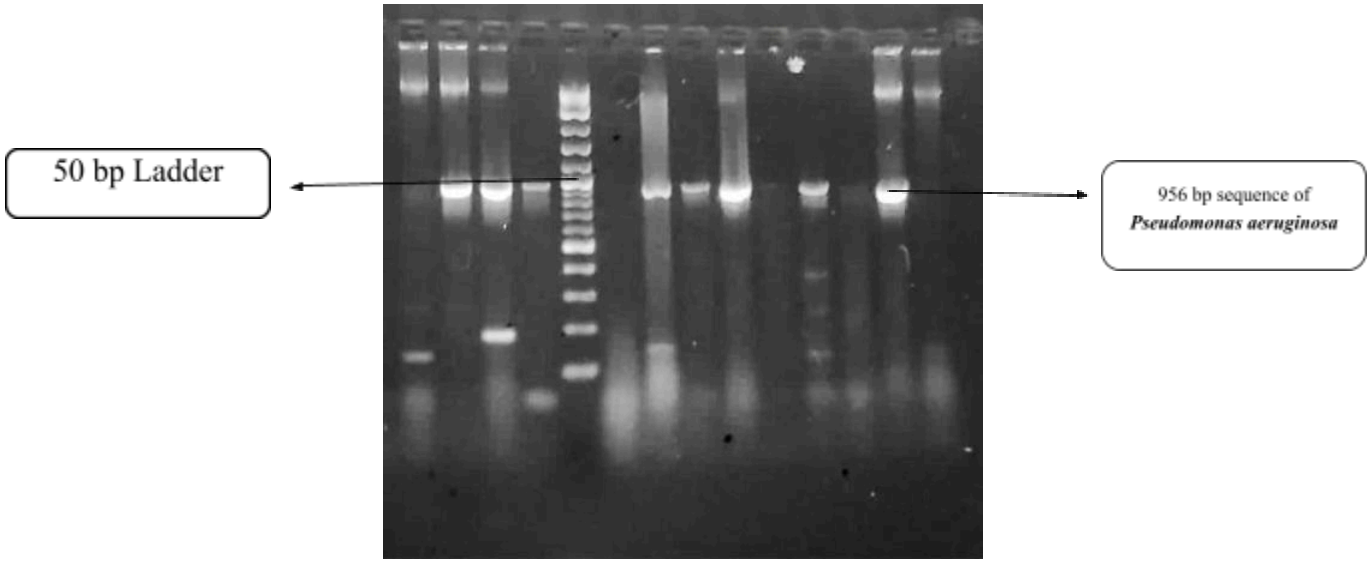

Fig. 8: PCR bands of *Pseudomonas aeruginosa.*

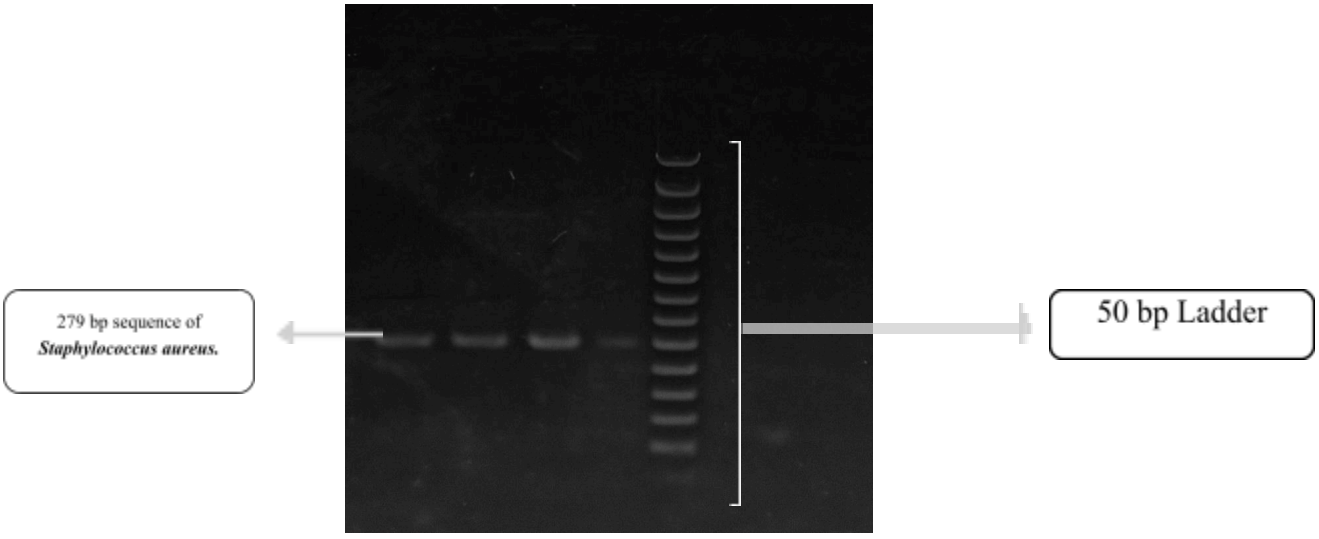

Fig. 9: PCR bands of *Staphylococcus aureus.*

4.2.3 Result Interpretation

The amplification of PCR confirmed the identities of two bacterial isolates. One of them is *Staphylococcus aureus,* and the other one is *Pseudomonas aeruginosa*. Therefore, the observation of clear bands on the agarose gel electrophoresis further confirmed the results. The NUC primer set was implemented to ensure the presence of *Staphylococcus aureus*. This set primarily targets the NUC gene, a distinctive identifier specifically designed for *Staphylococcus aureus*, which encodes a thermostable nuclease. The sequence of forward primers is 5’ GCG ATT GAT GGT GAT ACG GTT-3’, and the sequence of reverse primers is 5’ -ACG CAA GCC TTG ACG AAC TAA AGC-3’. Thus, the amplification of this process was seen to perform at an annealing temperature of 58° for each of the four samples out there. As it is, the primer combination here would be optimal. The analysis of PCR has revealed that a distinct unequivocal band was present across the four samples, which corresponds to the expected size of base pairs of 279. The band has recognized the isolates as *Staphylococcus aureus*. The PASS primer set was implemented to ensure the presence of *Pseudomonas aeruginosa.* This method is created especially for *Pseudomonas aeruginosa,* and it also manages to concentrate on a segment of the 16s ribosomal DNA (rDNA). In this, the sequence of forward primers is 5’-GGGGGATCT CGGACCTCA-3’, and the sequence of reverse primers is 5’-TCCATTATAGT GCCCACCCG-3’. The whole process of this reaction happened at a temperature of 58°. Eight of the samples demonstrated a distinct band at 956 BP in the image of a gel. The product size, as determined by the observation, managed to align with predictions, thereby confirming that the isolate is *Pseudomonas aeruginosa.* A DNA ladder of 50 bp was identified. The fragments here are in length, spaced in 50 bp increments, and consistently migrate across the gel at the same rate on each run. The bands of the PCR products, counting 279 bp for *S. aureus* and 956 bp for *P. aeruginosa,* managed to get into a comparison of the distances that were travelled by the sample bands.

4.3 Antibiotic Susceptibility Test (AST)

Antibiotic susceptibility testing is an important microbiological technique used to determine the effectiveness of different antimicrobial agents against specific bacterial infections. Healthcare professionals must leverage this information to select the most suitable antibiotic treatment, thereby aiding patient recovery and mitigating the emergence of antibiotic resistance.

4.3.1 Observation of AST

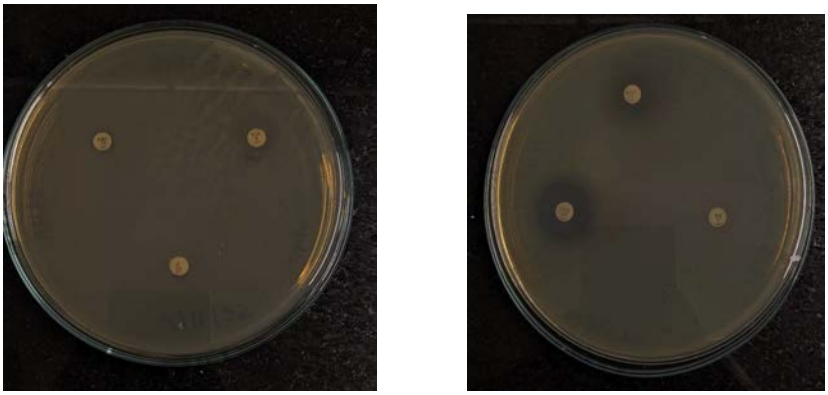

Fig. 10: AST results for selected antibiotics against *Pseudomonas aeruginosa*.

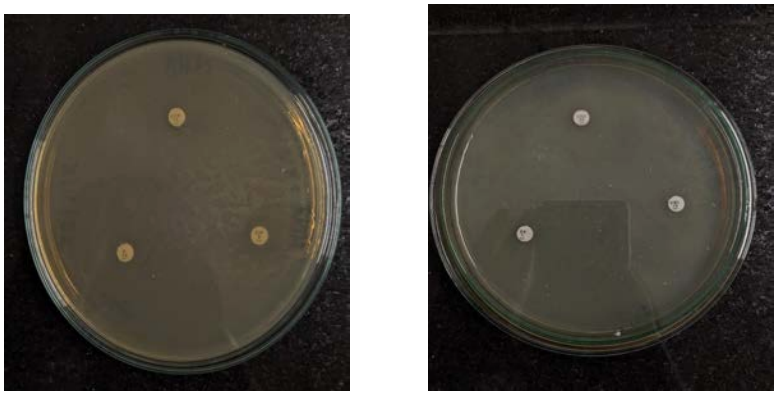

Fig. 11: AST results for selected antibiotics against *Staphylococcus aureus.*

4.3.2 Result Interpretation

The susceptibility profile indicates a notable degree of antimicrobial resistance in the tested isolates. As shown in the table, the Gram-positive organism *Staphylococcus aureus* demonstrated resistance to most of the antibiotics used in this study. The isolate was resistant to Amoxicillin (AML, 10 µg), Ceftriaxone (CRO, 30 µg), Azithromycin (AZM, 15 µg), Kanamycin (K, 30 µg), and Oxacillin (OX, 1 µg), while remaining susceptible only to Ciprofloxacin (CIP, 5 µg). In contrast, the Gram-negative organism *Pseudomonas aeruginosa* displayed a different susceptibility pattern. This isolate showed resistance to Amoxicillin (AML, 10 µg) and Kanamycin (K, 30 µg), which is consistent with the organism's well-documented intrinsic resistance mechanisms. However, it exhibited clear susceptibility to Ciprofloxacin (CIP, 5 µg) and Azithromycin (AZM, 15 µg), indicating that these agents remain effective treatment options for this strain. Oxacillin (OX, 1 µg) showed no inhibitory effect on *P. aeruginosa*, not due to acquired resistance, but because this antibiotic targets Gram-positive cell wall structures and is not active against Gram-negative bacteria.

4.4 Minimum Inhibitory Concentration (MIC) Test

The quantitative assessment of antimicrobial susceptibility, most commonly represented as the minimum inhibitory concentration (MIC), provides a definitive measure of the lowest concentration of an antibiotic, food-derived compound, or vitamin C required to inhibit visible microbial growth under controlled laboratory conditions. The broth microdilution method, along with comparable standardized approaches, generates continuous and precise MIC data that support the classification of bacterial isolates according to established clinical breakpoints as susceptible, intermediate, or resistant. Advances in MIC determination now include spectrophotometric analysis, which offers greater objectivity and reproducibility compared to traditional visual inspection. The resulting absorbance–concentration profiles are essential for interpreting inhibitory kinetics and for evaluating potential synergistic or antagonistic interactions in combination therapy studies, thereby contributing to a more accurate understanding of antimicrobial effectiveness (Hannan, 2020).

4.4.1 Observation of MIC Test

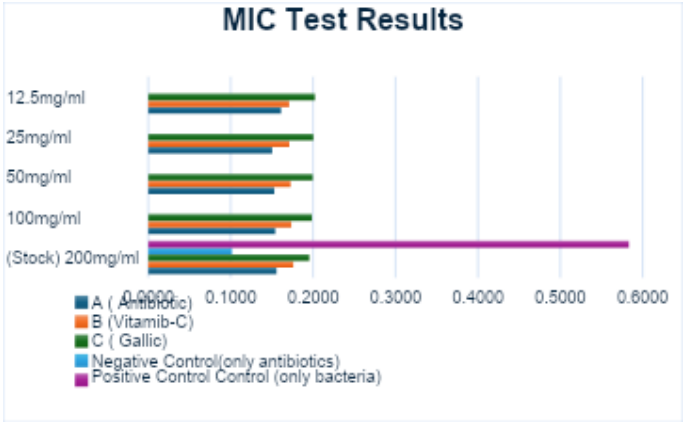

Fig. 12: MIC graphical results of *Staphylococcus aureus* against vitamin C, gallic acid, and ciprofloxacin.

4.4.2 MIC Test Interpretation

The graphical representation of the MIC test illustrates absorbance values along the x-axis and the corresponding concentrations of the tested samples, vitamin C, gallic acid, and ciprofloxacin, along the y-axis. The positive control (*Staphylococcus aureus* with no inhibitory agent) produced a markedly high absorbance reading, indicating strong bacterial growth and confirming that the organism remained viable throughout the experiment. In contrast, the negative control (medium containing only the antibiotic) exhibited very low absorbance, indicating the absence of bacterial growth and validating the reliability of the assay. The plotted results for each test molecule show a clear inverse relationship between concentration and bacterial growth, where increasing sample concentration is associated with progressively lower absorbance values. Based on this trend, the minimum inhibitory concentration (MIC) for the tested compounds in this experiment was determined to be 12.5 mg/ml, representing the lowest concentration at which visible bacterial growth was fully inhibited.

4.5 Ciprofloxacin, Vitamin C & Food ingredients against *Staphylococcus aureus*

This study investigates the interactions between the antibiotic ciprofloxacin and two food-derived compounds, gallic acid and vanillin, against *Staphylococcus aureus* . The objective is to determine whether these natural compounds enhance the activity of ciprofloxacin (synergistic effect), diminish its inhibitory action (antagonistic effect), or exert no measurable influence on its performance (neutral interaction), as described by Schuetz et al. (2025). The disc diffusion assay was employed as the primary method for assessment, with the diameter of the zone of inhibition serving as the key parameter for evaluating changes in antimicrobial effectiveness.

This region is the vacant area surrounding a paper disc that contains the test chemical and inhibits bacterial growth. An increased diameter indicates a more potent antibacterial effect. The experimental design evaluates various ratios of ciprofloxacin to the food molecule (such as 4:6 or 3:7) and incorporates essential control groups: the antibiotic alone (Con. Antibiotic), the food

molecule solely (Con. Food Molecule), vitamin C solely (Con. Vitamin C), and the primary test mixture (Con. Combination).

4.5.1 Observation of Combined Effect of Ciprofloxacin, Vitamin C, and Food Ingredients against *Staphylococcus aureus*

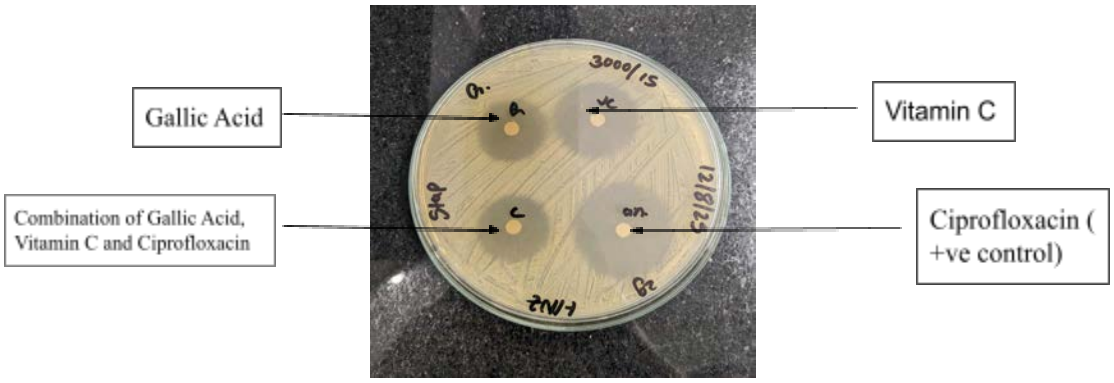

Fig 13: Combined Effect of Ciprofloxacin, Vitamin C and Gallic acid against *Staphylococcus aureus* at 3000 µg/15 µl concentration

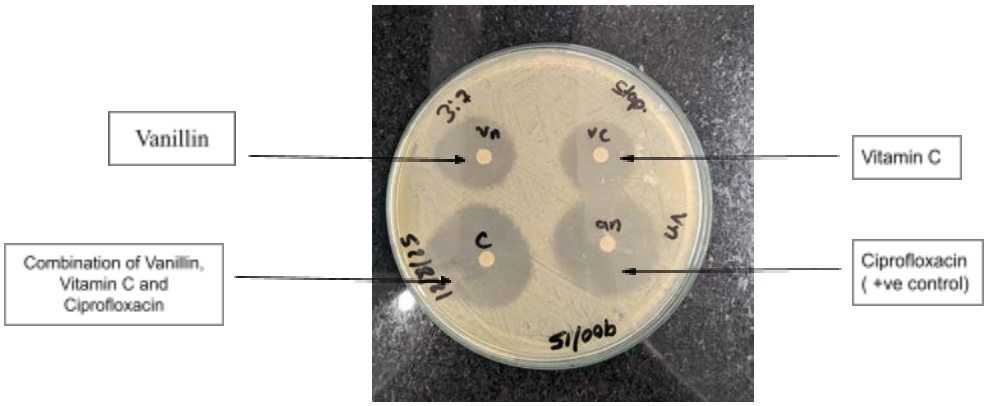

Fig 14: Combined Effect of Ciprofloxacin, Vitamin C and Vanillin against *Staphylococcus aureus* at 900 µg/15 µl concentration

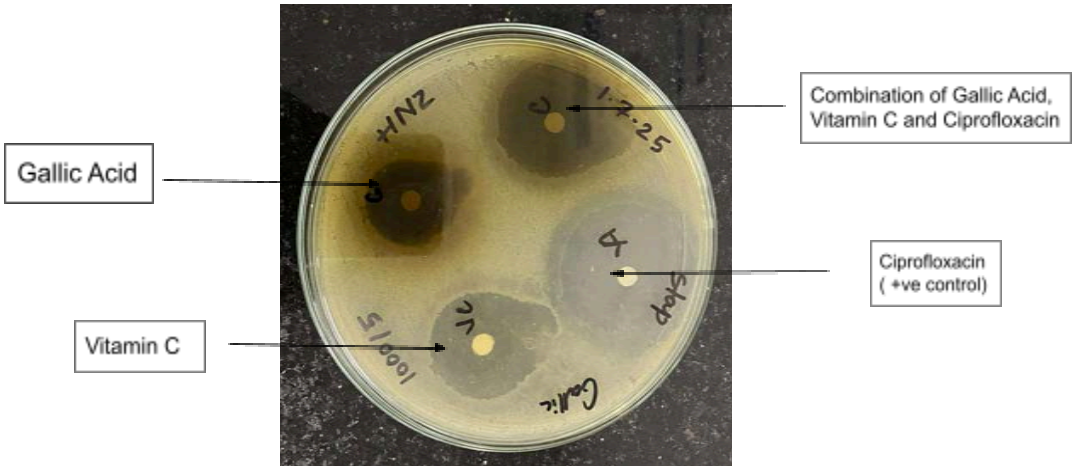

Fig 15: Combined Effect of Ciprofloxacin, Vitamin C and Gallic acid against *Staphylococcus aureus* at 1000 µg/5 µl concentration

4.5.2 Table 06: Combined Ciprofloxacin, Vitamin C, and Food Ingredients against *Staphylococcus aureus:*

Serial no:	Microorganisms:	2 g/10 ml ratios	Con. Vitamin C (µg/ µl) A	Con. Food Molecule (µg/ µl B	Con. Antibiotic(µg/ µl) C	Con. Combination(µg / µl0 (A + B + C) / 3 (µg/µl)	Zone of inhibition of Vitamin C (µg/µl)	Zone of inhibition of the food molecule (µg/µl)	Zone of inhibition of antibiotic (µg/µl)	Zone of inhibition of the combiantiontion (µg/µl)
Ciprofloxacin with gallic acid										
1	*S. aureus*	4:6	400 µg/5 µl	400 µg/5 µl	10 µg/5 µl	270 µg/5 µl	0 mm	15 mm	37 mm	18 mm
2	*S. aureus*	3:7	300 µg/5 µl	300 µg/5 µl	10 µg/5 µl	203 µg/5 µl	9 mm	10 mm	34 mm	20 mm
3	*S. aureus*	2:0	1000 µg/5 µl	1000 µg / 5 µl	10 µg/5 µl	670 µg/5 µl	12 mm	23 mm	40 mm	32 mm
4	*S. aureus*	4:6	1200 µg/1515 µl	1200 µg / 15 µl	30 µg/15 µl	810 µg/15 µl	15 mm	28 mm	39 mm	35 mm
5	*S. aureus*	3:7	900 µg/15 µl	900 µg/15 µl	30 µg/15 µl	610 µg/15 µl	inhibition	0 mm	37 mm	30 mm
6	*S. aureus*	2:0	3000 µg/15 µl	3000 µg / 15 µl	3000 µg/15 µl	2010 µg/15 µl	22 mm	33 mm	38 mm	40 mm
Ciprofloxacin with Vanillin										
1	*S. aureus*	4:6	400 µg/5 µl	400 µg/5 µl	10 µg/5 µl	270 µg/5 µl	0 mm	8 mm	39 mm	27 mm
2	*S. aureus*	3:7	300 µg/5 µl	300 µg/5 µl	10 µg/5 µl	203 µg/5 µl	5 mm	8 mm	37 mm	28 mm
3	*S. aureus*	2:0	1000 µg/5 µl	1000 µg / 5 µl	10 µg/5 µl	670 µg/5 µl	11 mm	10 mm	37 mm	30 mm

4	*S. aureus*	4:6	1200 µg/1515 µl	1200 µg / 15 µl	30 µg/15 µl	810 µg/1 5 µl	14 mm	12 mm	39 mm	32 mm
5	*S. aureus*	3:7	900 µg/15 µl	900 µg/15 µl	30 µg/15 µl	610 µg/15 µl	15 mm	17 mm	37 mm	30 mm
6	*S. aureus*	2:0	3000 µg/1515 µl	3000 µg / 15 µl	3000 µg/15 µl	2010 µg/15 µl	18 mm	20 mm	39 mm	inhibitio n

4.5.4 Combination of Ciprofloxacin, Vitamin C and Food Ingredients against *Staphylococcus aureus* Test Results Interpretation

The combination assays against *Staphylococcus aureus* showed a mixed interaction pattern, with evidence of both antagonistic and synergistic effects depending on the ratio and total load of the added compounds. When ciprofloxacin was combined with vitamin C and gallic acid at 5 µL volume, most combinations produced smaller zones than ciprofloxacin alone, indicating partial loss of activity. For example, at a 4:6 ratio (400 µg/5 µL of vitamin C and 400 µg/5 µL of gallic acid, plus 10 µg/5 µL ciprofloxacin), the combination gave an 18 mm zone compared with 37 mm for ciprofloxacin alone. At 3:7 (300 µg/5 µL vitamin C + 300 µg/5 µL gallic acid + 10 µg/5 µL ciprofloxacin) the zone was 20 mm versus 34 mm, and at 2:0 (1000 µg/5 µL gallic acid; 10 µg/5 µL ciprofloxacin) the combination reached 32 mm against a 40 mm control. These reductions show that at lower volumes and intermediate ratios, the interaction tends to be indifferent to mildly antagonistic, with ciprofloxacin still dominant.

When the volume was increased to 15 µL, the pattern became more nuanced. At 4:6 (1200 µg/15 µL of vitamin C and 1200 µg/15 µL of gallic acid, 30 µg/15 µL ciprofloxacin), the combination produced a 32 mm zone versus 39 mm for the antibiotic alone, and at 3:7 (900 µg/15 µL + 900 µg/15 µL; 30 µg/15 µL ciprofloxacin), the halo measured 30 mm compared with 37 mm. Both of these still reflect partial antagonism or at best a supportive but not enhancing effect. However, at the highest gallic acid load (2:0 ratio, 3000 µg/15 µL; 30 µg/15 µL ciprofloxacin), the combination reached 40 mm, while ciprofloxacin alone measured 38 mm. This slightly larger zone shows a clear synergistic event, where the high gallic acid concentration appears to contribute additional antibacterial pressure on top of ciprofloxacin.

The vanillin combinations against *S. aureus* were more clearly antagonistic or neutral. At 5 µL, ratios of 4:6 and 3:7 (400 or 300 µg/5 µL vitamin C, 400 or 300 µg/5 µL vanillin, 10 µg/5 µL ciprofloxacin) produced combination zones of 27–28 mm, whereas ciprofloxacin alone gave 37–39 mm. At 15 µL, the

4:6 and 3:7 trials (1200 or 900 µg/15 µL plus 30 µg/15 µL ciprofloxacin) yielded 30–32 mm halos compared with 37–39 mm for the antibiotic control. In the highest-dose 2:0 vanillin condition at 3000 µg/15 µL, ciprofloxacin still formed a 39 mm zone, but the combination disc failed to produce a measurable halo, indicating complete functional antagonism at that loading. Thus, for *S. aureus*, ciprofloxacin with vitamin C and gallic acid shows both antagonistic and occasional synergistic effects depending on dose, whereas ciprofloxacin with vanillin is predominantly antagonistic, often reducing or abolishing the antibiotic's observable activity.

4.6 Ciprofloxacin, Vitamin C & Food ingredients against *Pseudomonas aeruginosa*

his research study investigates the potential synergistic or antagonistic antibacterial effect of combining food-derived ingredients, particularly gallic acid and vanillin, with vitamin C and the antibiotic ciprofloxacin in order to combat MDR bacteria *Pseudomonas aeruginosa*. Here the disc or well diffusion method is used. This is as it allows a swift qualitative evaluation of the efficacy of the molecules in inhibiting MDR bacterial growth. The distinct and quantifiable inhibition zones generated by each treatment serve as the primary indicators of antimicrobial effectiveness, both for individual compounds and for their combined formulations. A major strength of the experimental design lies in the use of multiple concentration ratios for each component, which allows for a systematic evaluation of how varying proportions of antibiotic, vitamin C, and food-derived ingredients influence bacterial inhibition. These gradient-based data points are essential for identifying whether true synergistic interactions occur, as well as for determining the specific combinations that yield the most favorable inhibitory outcomes. The significance of this study extends beyond basic antimicrobial assessment, as it examines naturally occurring compounds as potential adjuvants that could enhance the activity of standard antibiotics. Such investigations are particularly important in the context of rising antimicrobial resistance, especially in highly resilient pathogens such as *Pseudomonas aeruginosa*. The dataset presented here outlines the inhibitory patterns of each individual agent and their mixtures, providing the foundation for interpreting whether any combination demonstrates genuine synergy rather than simple additive or antagonistic effects. A comparative analysis will be conducted on the zones of inhibition for the combinations in contrast to those of the antibiotic

alone and other individual agents to ascertain whether the combination therapy or treatment has a significantly enhanced antibacterial effect. This study may signify a novel strategy to address infections that demonstrate resistance to antibiotics.

4.6.1 Observation of Ciprofloxacin against *Pseudomonas aeruginosa*

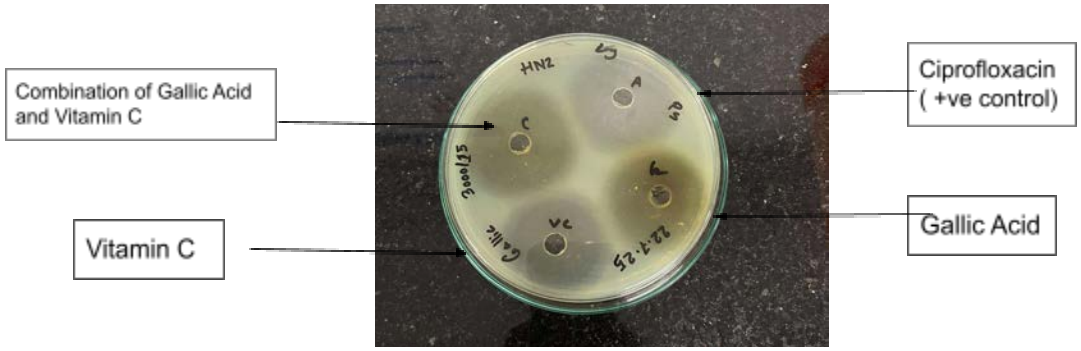

Fig. 16: Combined Effect of Ciprofloxacin vitamin C and gallic acids against *Pseudomonas aeruginosa* at 3000 µg/15 µl

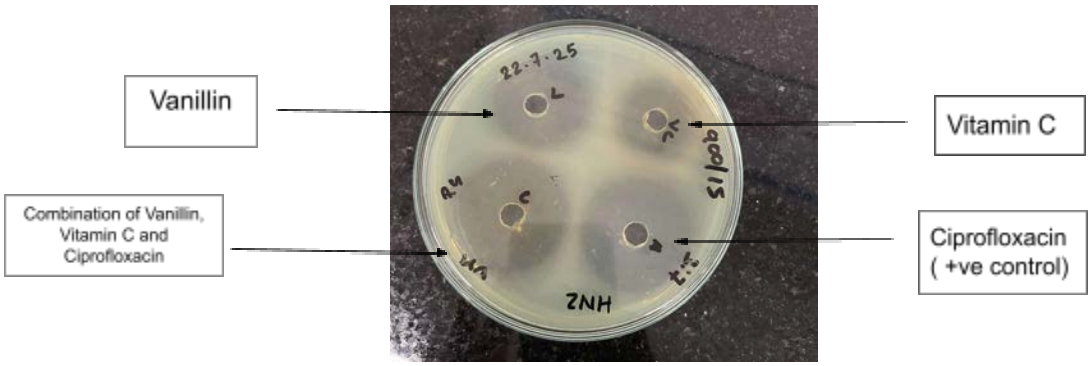

Fig. 17: Combined Effect of Ciprofloxacin, Vitamin C, and Vanillin against *Pseudomonas aeruginosa* at 900 µg/15 µl.

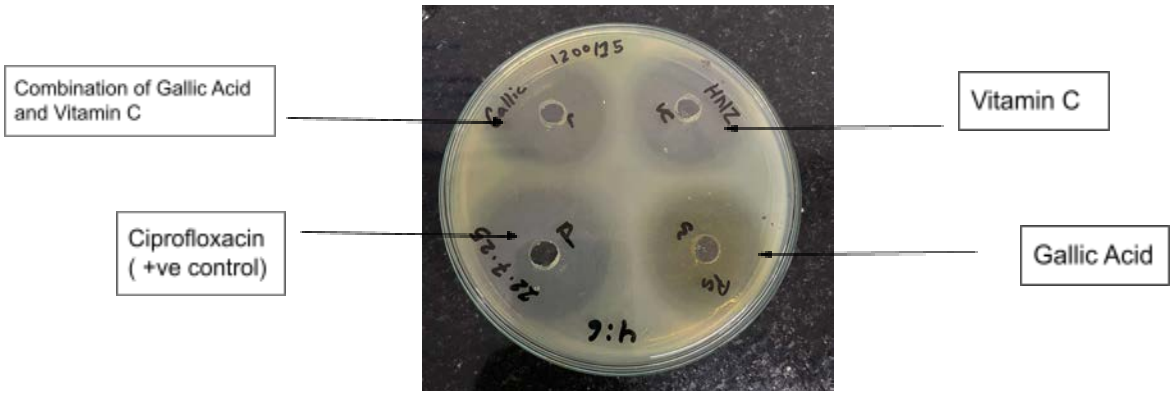

Fig 18: Combined Effect of ciprofloxacin, vitamin C, and gallic acid against *Pseudomonas aeruginosa* at 1200 µg/15 µl.

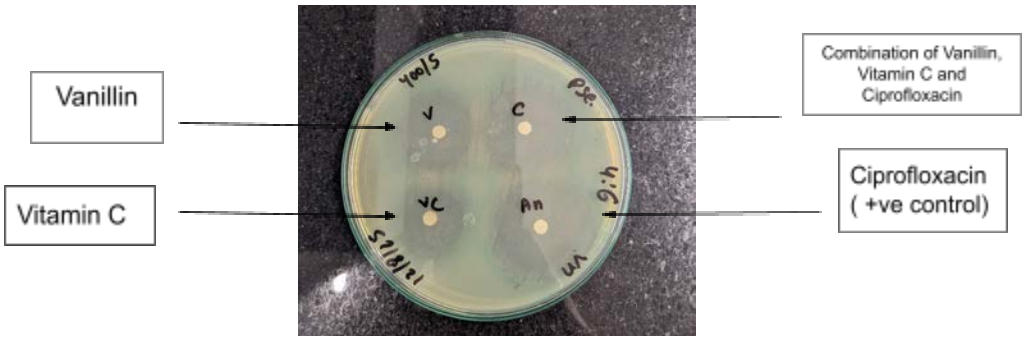

Fig 19: Combined Effect of Ciprofloxacin, Vitamin C, and Vanillin against *Pseudomonas aeruginosa* at 400 µg /15 µl.

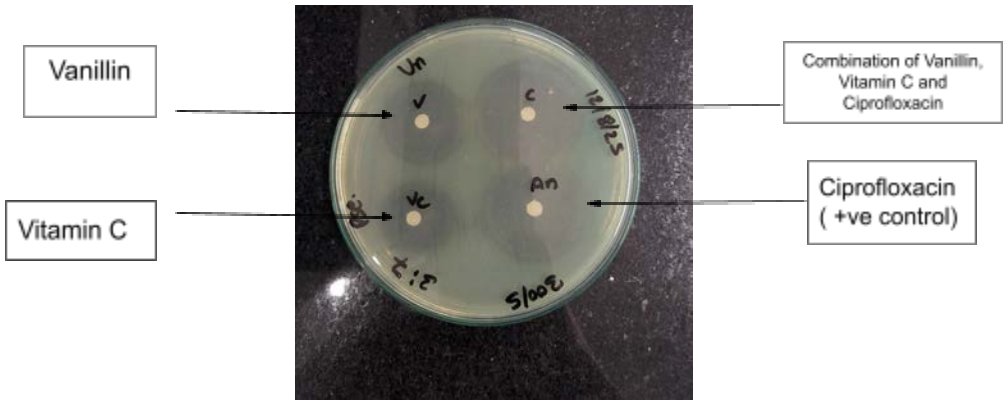

Fig 20: Combined Effect of Ciprofloxacin, Vitamin C, and Vanillin against *Pseudomonas aeruginosa* at 300 µg/15 µl.

4.6.2 Table 07: Combined Effect of Ciprofloxacin, Vitamin C, and Food Ingredients against *Pseudomonas aeruginosa*

Serial no:	Microorganisms:	2g/10ml Ratio	Con. Vitamin C: (µg/µl) A	Con. ingredient: (µg/µl) B	Con. Antibiotic:(µg/µl) C	Con. Combination: (A+B+C)/3 (µg/µl)	Zone of inhibition of Vimatin c: (µg/µl)	Zone of inhibition of food molecule (µg/µl)	Zone of inhibition of antibiotic) (µg/µl)	Zone of inhibition of the combination: (µg/µl)
Ciprofloxacin with Gallic										
1	*P.aeruginosa*	4:6	400 µg / 5 µl	400 µg/5 µl	10µg/5 µl	270 µg/5 µl	0 mm	0 mm	35mm	27mm
2	*P.aeruginosa*	3:7	300µg/ 5µl	300µg/5 µl	10µg/5 µl	203 µg/5 µl	0 mm	0 mm	32mm	23mm
3	*P.aeruginosa*	2:0	1000µg /5µl	1000µg/ 5µl	1000µg/5µl	670 µg/5 µl	0 mm	oxidised	38mm	31mm
4	*P.aeruginosa*	4:6	1200µg /15µl	1200µg/ 15µl	30µg/15µl	810 µg/15 µl	32mm	28mm (oxidised)	45mm	36mm
5	*P.aeruginosa*	3:7	900µg/ 15µl	900µg/15µl	30µg/15µl	610µg/15µl	28mm	23mm (oxidised)	43mm	34mm
6	*P.aaeruginosa*	2:0	3000µg /15µl	3000µg/ 15µl	30µg/15µl	2010µg/ 15µl	35mm	32mm (oxidised)	41mm	43mm

Ciprofloxacin with Vanillin										
1	*P.aeruginosa*	4:6	400 µg/5 µl	400 µg/5 µl	10 µg/5 µl	270 µg/5 µl	25 mm	29 mm	39 mm	36 mm
2	*P.aeruginosaaeruginosa*	3:7	300 µg/5 µl	300 µg/5 µl	10 µg/5 µl	203 µg/5 µl	22 mm	27 mm	38 mm	34 mm
3	*P. aeruginosa*	2:0	1000 µg/5 µl	1000 µg / 5 µl	10 µg/5 µl	670 µg/5 µl	27 mm	30 mm	39 mm	37 mm
4	*P.aeruginosa*	4:6	1200 µg/15 µl	1200 µg / 15 µl	30 µg/15 µl	810 µg/15 µl	32 mm	33 mm	43 mm	42 mm
5	*P.aserugin osa*	3:7	900 µg / 15 µl	900 µg/15 µl	30 µg/15 µl	610 µg/15 µl	28 mm	32 mm	42 mm	40 mm
6	*P.aeruginosa*	2:0	3000 µg/15 µl	3000 µg / 15 µl	30 µg/15 µl	2010 µg/15 µl	34 mm	38 mm	44 mm	43 mm

- **N/A: No activity**

4.6.3 Combination of Ciprofloxacin, Vitamin C, and Food Ingredients against *Pseudomonas aeruginosa* Interpretation

Against *Pseudomonas aeruginosa*, the interaction profile between ciprofloxacin, vitamin C, and the food-derived ingredients is again mixed but weighted toward indifferent or antagonistic effects, with only borderline supportive behavior at the highest concentrations. Ciprofloxacin alone consistently produced the largest zones, typically in the range of 32–45 mm, confirming it as the main active agent. When combined with vitamin C and gallic acid at 5 µL, the inhibition zones were consistently smaller than those of ciprofloxacin alone, which points to mild antagonism. At a 4:6 ratio (400 µg/5 µL vitamin C, 400 µg/5

µL gallic acid, 10 µg/5 µL ciprofloxacin), the combination zone was 27 mm compared with 35 mm for the antibiotic alone. At 3:7 (300 µg/5 µL + 300 µg/5 µL; 10 µg/5 µL ciprofloxacin), the halo dropped to 23 mm against a 32 mm control, and at 2:0 (1000 µg/5 µL gallic acid plus 10 µg/5 µL ciprofloxacin) the combination measured 31 mm while ciprofloxacin alone reached 38 mm. These reductions suggest that in *P. aeruginosa*, the added gallic acid and partly oxidised vitamin C tend to dampen rather than boost ciprofloxacin's effect at low volume. At 15 µL, the overall activity increased for all discs, yet the relative pattern remained similar. In the 4:6 and 3:7 gallic acid mixes (1200 and 900 µg/15 µL, with 30 µg/15 µL ciprofloxacin), the combinations produced 36 mm and 34 mm zones, whereas ciprofloxacin alone gave 45 mm and 43 mm, respectively, again indicating partial antagonism. The highest gallic acid loading (2:0 ratio, 3000 µg/15 µL plus 30 µg/15 µL ciprofloxacin) generated a 43 mm combination halo compared with 44 mm for ciprofloxacin alone. This near-identical size suggests a borderline additive or weakly synergistic effect, where the helper compounds do not clearly improve activity but also no longer substantially reduce it.

The vanillin combinations followed a comparable pattern. At 4:6 and 3:7 ratios with vanillin (for example, 400–300 µg/5 µL or 1200–900 µg/15 µL of vitamin C and vanillin with a fixed ciprofloxacin dose), the combination zones generally fell in the 34–42 mm range, while the corresponding ciprofloxacin controls ranged from 39–44 mm. Thus, vanillin-containing discs tended to produce zones that were slightly smaller but still close to the antibiotic alone. This behavior can be interpreted as mostly indifferent with a mild antagonistic shift, since no vanillin combination clearly exceeded the ciprofloxacin halo, though at the highest loads the difference became very small and functionally close to additive.

Taken together, the *P. aeruginosa* data show that ciprofloxacin remains the primary driver of inhibition, with vitamin C, gallic acid, and vanillin acting mainly as modulators. At several lower-dose combinations the interaction is antagonistic, reducing zone size, while at the upper concentration limit the mixtures approach the ciprofloxacin control and can be viewed as borderline additive or weakly synergistic.

4.7 Vitamin C and Food Ingredients against *Staphylococcus aureus*

This data table provides a distinctive and important compilation of control tests conducted to understand the antibacterial properties of the food-derived ingredients, vitamin C, and antibiotic used in the primary research. The main objective was to establish a negative control baseline, distinguishing it from the previous datasets that focused on active chemicals. The trials systematically evaluate a variety of food-derived ingredients, particularly varying ratios of gallic acid and vanillin, turmeric, and black seed, all prepared in the same volume and concentration, which is 2 g/10 ml & 1 g/10 ml in regard to *Staphylococcus aureus* (Stap). The initial

observation is that the concentration of the active ingredient is recorded at 0 µg, which is denoted as “NA” in nearly all conducted tests. This suggests that the use of food-derived molecules does not effectively contribute as antibacterial agents.

4.7.1 Observation of Vitamin C and Food Ingredients against *Staphylococcus aureus*

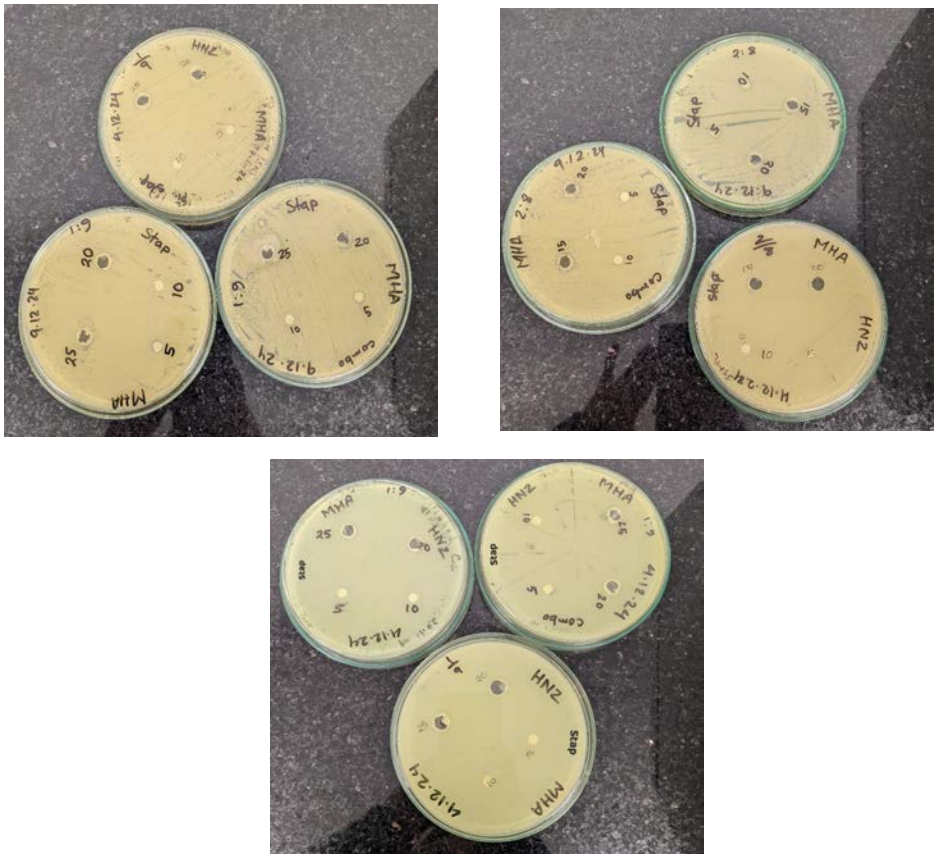

Fig 21: Combined effect of Vitamin C and Food Ingredients against *Staphylococcus aureus.*

4.7.2 Table 08: Combined Effect of Vitamin C and Food Ingredients against *Staphylococcus aureus*

Serial no:	Date of the Experiment:	Microorganisms:	Con. Vitamin C(µg/µl) A	Con. Food Molecule (µg/µl) B	Con. Combination(µg/µl) A+B/2 (µg/µl)	Zone of inhibition of Vitamin C(µg/µl)	Zone of inhibition of Food molecule:(µg/µl)	Zone of inhibition of the combination:(µg/µl)
1:9 ratio of 2g/10ml (Gallic Acid + Vanillin)								
1	10.11.24	*S. aureus*	100µg/5µl	100µg/5µl	100µg/5µl	N/A	N/A	N/A
2		*S. aureus*	200µg/10µl	200µg/10µl	200µg/10µl	N/A	N/A	N/A
3		*S. aureus*	300µg/15µl	300µg/15µl	300µg/15µl	N/A	N/A	N/A
4		*S. aureus*	400µg/20µl	400µg/20µl	400µg/20µl	N/A	N/A	N/A
2:8 ratio of 2g/10ml (Gallic Acid + Vanillin)								
	15.11.24	*S. aureus*	200µg/5µl	200µg/5µl	200µg/5µl	N/A	N/A	N/A
		S. aureus	400µg/10µl	400µg/10µl	400µg/10µl	N/A	N/A	N/A
		S. aureus	600µg/15µl	600µg/15µl	600µg/15µl	N/A	20mm	N/A

		S. aureus	800µg/20µl	800µg/20µl	800µg/20µl	N/A	29mm	22mm
2:8 ratio of 1g/10ml (Vanillin)								
1	09.12.24	*S. aureus*	100µg/5µl	100µg/5µl	100µg/5µl	N/A	N/A	N/A
2		*S. aureus*	200µg/10µl	200µg/10µl	200µg/10µl	N/A	N/A	N/A
3		*S. aureus*	300µg/15µl	300µg/15µl	300µg/15µl	N/A	N/A	N/A
4		*S. aureus*	400µg/20µl	400µg/20µl	400µg/20µl	N/A	N/A	N/A
1:9 ratio of 1g/10ml (Vanillin)								
1	09.12.24	*S. aureus*	50µg/5µl	50µg/5µl	50µg/5µl	N/A	N/A	N/A
2		*S. aureus*	100µg10/µl	100µg10/µl	100µg10/µl	N/A	N/A	N/A
3		*S. aureu*	150µg/15µl	150µg/15µl	150µg/15µl	N/A	N/A	N/A
4		*S. aureus*	250µg/25µl	250µg/25µl	250µg/25µl	N/A	N/A	N/A
2:8 ratio of 2g/10ml (Turmeric)								
1	29.12.24	*S. aureus*	200µg/5µl	200µg/5µl	200µg/5µl	N/A	N/A	N/A
2		*S. aureus*	400µg10/µl	400µg10/µl	400µg10/µl	N/A	N/A	N/A

3		*S. aureus*	600µg/15µl	600µg/15µl	600µg/15µl	N/A	N/A	N/A
4		*S. aureus*	800µg/20µl	800µg/20µl	800µg/20µl	N/A	N/A	N/A
1:9 ratio of 2g/10ml (Turmeric)								
1	29.12.24	*S. aureus*	100µg/5µl	100µg/5µl	100µg/5µl	N/A	N/A	N/A
2		*S. aureus*	200µg10/µl	200µg10/µl	200µg10/µl	N/A	N/A	N/A
3		*S. aureus*	400µg/20µl	400µg/20µl	400µg/20µl	N/A	N/A	N/A
4		*S. aureus*	500µg/25µl	500µg/25µl	500µg/25µl	N/A	N/A	N/A
2g/10ml (Black Seed)								
1	14.02.25	*S. aureus*	400µg/10µl	400µg/10µl	400µg/10µl	N/A	N/A	N/A
2		*S. aureus*	200µg/10µl	200µg/10µl	200µg/10µl	N/A	N/A	N/A
3		*S. aureus*	100µg/10µl	100µg/10µl	100µg/10µl	N/A	N/A	N/A

- **N/A: No Activity**

4.7.3 Vitamin C and Food Ingredients against *Staphylococcus aureus* Interpretation

The solvent controls for the gallic acid and vanillin mixtures demonstrate minimal antibacterial activity, indicating their effectiveness as negative controls. The results consistently show a complete lack of antibacterial activity across all experiments performed at 1:9 and 2:8 ratios

using the 2 g/10 mL stock concentrations of the food molecules, regardless of whether 5 µL, 10 µL, 15 µL, or 20 µL volumes were applied. In every case, the “Zone of Inhibition” values for Vitamin C, the individual food molecule, and their combined application remained recorded as “NA,” indicating no observable antimicrobial effect. This confirms that the solvent used to prepare the stock solutions does not inhibit the growth of *Staphylococcus aureus*. However, an exception was observed at higher concentrations: the combination of gallic acid and vanillin at 600 µg/15 µL produced a measurable 20 mm zone of inhibition. Furthermore, when applied individually at 800 µg/20 µL, both gallic acid and vanillin generated a 29 mm inhibition zone, while their combination with Vitamin C produced a 22 mm zone. In contrast, vanillin prepared at a lower concentration (1 g/10 mL) demonstrated no antibacterial activity, as all zones across all tested volumes remained recorded as “NA.”

The findings are unequivocal for both the 2:8 and 1:9 ratios of the 2 g/10 ml turmeric solution. The zones of inhibition consistently exhibit "NA" (No Activity) across all tested volumes: 5 µl, 10 µl, 15 µl, 20 µl, and 25 µl. This indicates that the solvent employed in the preparation of the turmeric stock does not exhibit lethal effects on Staphylococcus aureus. The control tests conducted for the Black Seed solution (2 g/10 ml) demonstrate a consistent trend. The results consistently show “NA” across all columns, despite the execution of two tests at a volume of 10 µl. The consistent occurrence reinforces the dependability of the null results.

4.8 Vitamin C and Food Ingredients against *Pseudomonas aeruginosa*

This study presents a structured series of experiments aimed at evaluating the antibacterial activity of vitamin C, gallic acid, vanillin, turmeric, black seed, and their various combinations against *Pseudomonas aeruginosa* (Pseudo). Unlike the previous sets of assays that incorporated ciprofloxacin, this section exclusively examines the ability of natural compounds individually and in blended formulations to inhibit bacterial growth. Multiple concentration ratios (1:9 and 2:8), spanning dosages from 50 µg to 800 µg and applied across different volumes, were systematically tested. Throughout the experiments, the “Zone of Inhibition” served as the principal indicator of antibacterial efficacy. This experimental framework provides a consistent basis for determining whether these natural agents possess intrinsic antimicrobial properties or

demonstrate enhanced activity when paired, especially against a pathogen known for its high level of treatment resistance.

4.8.1 Observation of Combination of Vitamin C and Food ingredient against *Pseudomonas aeruginosa*

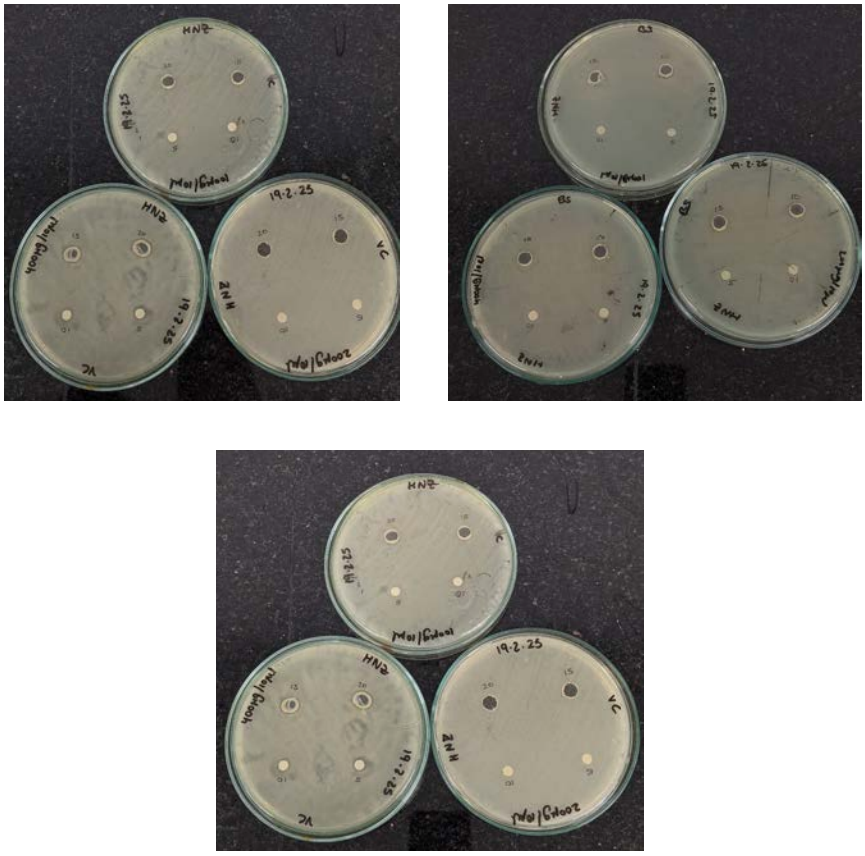

Fig 22: Combined Effect of Vitamin C and Food Ingredients against *Pseudomonas aeruginosa.*

4.8.2 Table 09: Combined Effect of Vitamin C and Food Ingredient against *Pseudomonas aeruginosa.*

Serial no:	Date of the Experiment:	Microorganisms:	Con. Vitamin C: (µg/µl) A	Con. Food Molecule: (µg/µl) B	Con. Combination: (µg/µl) C	Zone of inhibition of Vitamin C: (µg/µl) A+B+C/3 (µg/µl)	Zone of inhibition of Food molecule: (µg/µl)	Zone of inhibition of the combination: (µg/µl)
1:9 ratio of 2 g/10 ml (Gallic Acid)								
1	10.11.24	*P.aeruginosa*	100µg/5µl	100µg/5µl	100µg/5µl	N/A	N/A	N/A
2		*P.aeruginosa*	200µg/10µl	200µg/10µl	200µg/10µl	N/A	N/A	N/A
3		*P.aeruginosa*	300µg/15µl	300µg/15µl	300µg/15µl	N/A	N/A	N/A
4		*P.aeruginosa*	400µg/20µl	400µg/20µl	400µg/20µl	N/A	N/A	N/A
2:8 ratio of 2g/10ml								
1	15.11.24	*P.aeruginosa*	200µg/5µl	200µg/5µl	200µg/5µl	N/A	N/A	N/A
2		*P.aeruginosa*	400µg/10µl	400µg/10µl	400µg/10µl	N/A	N/A	N/A
3		*P.aeruginosa*	600µg/15µl	600µg/15µl	600µg/15µl	N/A	N/A	N/A
4		*P.aeruginosa*	800µg/20µl	800µg/20µl	800µg/20µl	N/A	N/A	N/A
2:8 ratio of 1g/10ml (Vanillin)								
1	04.12.24	*P.aeruginosa*	100µg/5µl	100µg/5µl	100µg/5µl	N/A	N/A	N/A

2		*P.aeruginosa*	200µg/10µl	200µg/10µl	200µg/10µl	N/A	N/A	N/A
3		*P.aeruginosa*	300µg/15µl	300µg/15µl	300µg/15µl	N/A	N/A	N/A
4		*P.aeruginosa*	400µg/20µl	400µg/20µl	400µg/20µl	N/A	N/A	N/A
1:9 ratio of 1g/10ml								
1	04.12.24	*P.aeruginosa*	50µg/5µl	50µg/5µl	50µg/5µl	N/A	N/A	N/A
2		*P.aeruginosa*	100µg10/µl	100µg10/µl	100µg10/µl	N/A	N/A	N/A
3		*P.aeruginosa*	150µg/15µl	150µg/15µl	150µg/15µl	N/A	N/A	N/A
4		*P.aeruginosa*	200µg/20µl	200µg/20µl	200µg/20µl	N/A	N/A	N/A
2:8 ratio of 2g/10ml (Turmeric)								
1	29.12.24	*P.aeruginosa*	200µg/5µl	200µg/5µl	200µg/5µl	N/A	N/A	N/A
2		*P.aeruginosa*	400µg10/µl	400µg10/µl	400µg10/µl	N/A	N/A	N/A
3		*P.aeruginosa*	600µg/15µl	600µg/15µl	600µg/15µl	N/A	N/A	N/A
4		*P.aeruginosa*	800µg/20µl	800µg/20µl	800µg/20µl	N/A	N/A	N/A
1:9 ratio of 2g/10ml (Turmeric)								
1	29.12.24	*P.aeruginosa*	100µg/5µl	100µg/5µl	100µg/5µl	N/A	N/A	N/A

2		*P.aeruginosa*	200µg10/µl	200µg10/µl	200µg10/µl	N/A	N/A	N/A
3		*P.aeruginosa*	400µg/20µl	400µg/20µl	400µg/20µl	N/A	N/A	N/A
4		*P.aeruginosa*	500µg/25µl	500µg/25µl	500µg/25µl	N/A	N/A	N/A
2g/10ml (Black Seed)								
1	14.02.25	*P.aeruginosa*	400µg/10µl	400µg/10µl	400µg/10µl	N/A	N/A	N/A
2		*P.aeruginosa*	200µg/10µl	200µg/10µl	200µg/10µl	N/A	N/A	N/A
3		*P.aeruginosa*	100µg/10µl	100µg/10µl	100µg/10µl	N/A	N/A	N/A

- **N/A: No Activity**

4.8.3 Vitamin C & Food Ingredients against *Pseudomonas aeruginosa* Interpretation:

The findings for gallic acid and vanillin, tested both independently and in combination with vitamin C, demonstrate a complete absence of antimicrobial activity against *Pseudomonas aeruginosa*. In all experiments using gallic acid at 2 g/10 mL, applied in 1:9 and 2:8 ratios and across doses ranging from 100 µg/5 µL to 800 µg/20 µL, no zone of inhibition was detected. All recorded values for the vitamin C control, gallic acid alone, and the gallic acid–vitamin C combination were consistently marked as "N/A," confirming that none of these treatments displayed inhibitory effects on *P. aeruginosa*. Vanillin showed an identical trend. At both 1 g/10 mL and 2 g/10 mL concentrations, and across every tested ratio and dose (50 µg/5 µL to 400 µg/20 µL), each measurement was recorded as "N/A." Although vanillin exhibited antimicrobial activity against *Staphylococcus aureus* in earlier experiments, these results verify that it lacks efficacy against *P. aeruginosa*, even when combined with vitamin C.

Turmeric and black seed displayed the same pattern of inactivity. Turmeric at 2 g/10 mL produced no measurable inhibition zone under either 1:9 or 2:8 conditions, regardless of dose (100 µg/5 µL to 800 µg/20 µL), with all values likewise reported as "N/A." This outcome indicates that turmeric, despite its known bioactive compounds, does not suppress the growth of *P. aeruginosa* within the tested concentration range. Black seed, evaluated at 2 g/10 mL across three doses (100 µg, 200 µg, and 400 µg applied in 10 µL volumes), also showed no antimicrobial effect. The vitamin C control, black seed alone, and the combination treatment all resulted in "N/A," reinforcing the conclusion that black seed extract does not inhibit *P. aeruginosa* under the defined experimental conditions.

Chapter 5: Discussion

5.1 Discussion

Multidrug resistance is rising in both gram-negative and gram-positive bacteria. While Gram-positive bacteria, such as *Staphylococcus aureus*, commonly acquire resistance via β-lactamase production and target modification, Gram-negative organisms, such as *Pseudomonas aeruginosa*, possess an intrinsic resistance provided by efflux pumps and poor membrane permeability (Fair & Tor, 2014). Rapid and excessive use of antibiotics accelerated the development of resistance, which has led to fewer treatment options and more clinical failures (Blair et al., 2015).

In this study, the antibiotic susceptibility test and molecular analysis provided crucial information about the genetic identity and resistance profile of the bacterial strains. In the AST, the two bacterial species showed different resistance patterns. *Staphylococcus aureus* showed resistance to Amoxicillin, Ceftriaxone, Azithromycin, Kanamycin, and Oxacillin. Methicillin-Resistant Staphylococcus aureus (MRSA) is characterized by resistance to oxacillin, which is mediated by the mecA gene encoding the modified penicillin-binding protein (PBP2a).The production of β-lactamases, which hydrolyze penicillins and many cephalosporins and make medications like amoxicillin and ceftriaxone ineffective, is one of the main reasons *Staphylococcus aureus* is resistant to many broad-spectrum antibiotics. Additionally, the bacterium forms biofilms, which produce metabolic and physical barriers that lessen drug activity and antibiotic penetration. However, *P. aeruginosa* showed resistance to only Amoxicillin and Kanamycin and were susceptible towards Ceftriaxone, Azithromycin and Ciprofloxacin. *P. aeruginosa,* however*,* is a very tough bacteria to treat since it has multiple resistance mechanisms, making many antibiotics ineffective. But the strain used in our study probably lacks the high-level resistance mechanism, suggesting low AmpC beta-lactamase expression and decreased low efflux pump of the strain. On the other hand, ciprofloxacin was effective against both *S. aureus* and *P. aeruginosa.* Ciprofloxacin, a second-generation fluoroquinolone, has been working efficiently for both Gram-positive and -negative bacteria by blocking DNA gyrase and topoisomerase IV. Several studies support our results. For instance, according to a study by A.A.H. Al Qushawi and K. J. Al-Ruaby in 2021, The disc diffusion technique was used to assess the antibiotic susceptibility of 54 *Pseudomonas aeruginosa*

bacterial isolates, and the findings indicated that ciprofloxacin was the most effective antibiotic against the bacterial isolates.

The PCR results provided strong molecular confirmation of both the strains. It gave the expected size and defined bands at 956 bp for *P. aeruginosa* and 279 bp for *S. aureus*. It confirms that successful amplification was achieved and thus supports the accuracy of the phenotypic identification done earlier. The gel results showed no evidence of extra bands or any primer dimer, which means there was no non-specific amplification, and it can be concluded that the reaction parameters, such as primer design, annealing temperature, and DNA extraction quality, were effectively optimized. Crucially, no amplification was seen outside of the target regions. indicating that cross-reaction and contamination were successfully managed during PCR setup. Overall, the gels show that the primers were very specific, the cycling parameters were correctly calibrated for clean amplification, and the DNA extracts were intact. However, the band intensity differed between lanes in *P. aeruginosa,* which might be because of inexact pipetting volume. A very light smearing is also seen in the lower portion of the *P. aeruginosa gel,* which could be caused by residual salts or DNA shearing. Regardless, the PCR results gave robust results with little technical imperfections, which is within the acceptable range.

This research explores the interactions between two food-derived compounds, such as gallic acid and vanillin (4-hydroxy-3-methoxybenzaldehyde), and the fluoroquinolone antibiotic ciprofloxacin in relation to their impact on *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Contrary to the original idea of synergistic potential, the disc diffusion study consistently showed a largely antagonistic relationship, wherein the addition of either natural component often reduced ciprofloxacin's antibacterial activity. The main focus of this work was to explore how food derived compounds, particularly gallic acid and vanillin (4-hydroxy-3-methoxybenzaldehyde), interact with ciprofloxacin against *S. aureus* and *P. aeruginosa*. All natural compounds were prepared from a stock solution containing 2 g in 10 mL and then diluted with distilled water in ratios of 4:6, 3:7, and 2:0 (stock:water). This produced working loads between roughly 300 µg in 5 µL and 3000 µg in 15 µL on each disc. The concentrations of vitamin C, gallic acid or vanillin, and ciprofloxacin were then averaged for the different combination sets so that direct comparisons could be made. At these loads, vitamin C

and gallic acid showed measurable antibacterial activity on their own against *S. aureus*, whereas vanillin and all three compounds against *P. aeruginosa* behaved as relatively weak direct agents and required the highest microgram doses to produce clear zones.

When combined with ciprofloxacin, the interaction pattern was not uniform. In several *S. aureus* combinations, particularly those containing vitamin C and gallic acid at 4:6 and 3:7 from the 2 g per 10 mL stock, the zones of inhibition were larger than those produced by ciprofloxacin alone. For example, sample 6 produced a 40 mm zone compared with a 38 mm zone for ciprofloxacin on the same plate, which indicates a modest but real synergistic effect under those conditions. In *P. aeruginosa*, some combination discs also approached the ciprofloxacin control and in a few cases slightly exceeded the individual components, again suggesting that certain high gallic acid mixtures can act synergistically. At the same time, many other fixed ratio mixtures, especially those richer in vanillin or with very high total phenolic load, produced zones that were equal to or smaller than ciprofloxacin alone. In both organisms, several sets fell in this category, giving patterns best described as indifferent or mildly antagonistic. Thus, the data support the presence of both synergistic and antagonistic interactions within the same experimental design, depending strongly on the ratio and concentration of the natural compounds relative to ciprofloxacin.

The difference in zone sizes between *S. aureus* and *P. aeruginosa* also appears to reflect their contrasting cell envelope structures. *S. aureus* has a thick, multilayered peptidoglycan cell wall that acts as a robust barrier and can hinder the passage of both hydrophilic and hydrophobic agents (Silhavy et al., 2010). *P. aeruginosa*, like other Gram negative bacteria, has a thin peptidoglycan layer located between an inner membrane and an outer membrane rich in lipopolysaccharides. The outer membrane restricts certain antibiotics, but once compounds pass through porin channels the thin peptidoglycan offers relatively little resistance (Delcour, 2009). This architecture helps explain why ciprofloxacin and some phenolic derivatives showed stronger or more consistent activity against *P. aeruginosa* in several combinations, while *S. aureus* often required higher loads to reach comparable effects.

Ciprofloxacin primarily targets DNA gyrase (topoisomerase II) and topoisomerase IV, which are essential for DNA replication, supercoiling control, and chromosome segregation (Hooper & Jacoby, 2016). *In S. aureus*, topoisomerase IV is usually the main target, but overall activity can

be limited by the thick cell wall and efflux systems such as NorA, which reduce intracellular drug accumulation (Kaatz et al., 1993). In *P. aeruginosa*, DNA gyrase remains the principal target. Once ciprofloxacin crosses the outer membrane through porins such as OprF, it can accumulate effectively despite the presence of efflux pumps, especially at the concentrations used in this study. These species specific differences in target accessibility and cell penetration likely contribute to the variations seen in the combination assays.

Vitamin C, gallic acid, and vanillin each act on bacteria through different mechanisms. Vitamin C (ascorbic acid) can generate reactive oxygen species, disturb redox balance, and destabilise membranes by promoting lipid peroxidation (Vilchéze et al., 2018). Gallic acid, a phenolic acid, can insert into membranes, increase permeability, chelate metal ions, and inhibit key enzymes, leading to cytoplasmic leakage and metabolic disruption (Borges et al., 2013). Vanillin, an aromatic aldehyde, has been reported to interfere with cell wall integrity, quorum sensing, and oxidative balance (Nazzaro et al., 2013). Once these molecules pass through porins, they may traverse gram negative cell envelopes relatively easily, whereas the thick peptidoglycan of gram positive bacteria poses a greater barrier. When combined with ciprofloxacin, these effects can either favour or hinder antibiotic action. Increased membrane permeability and surface destabilisation by vitamin C and gallic acid may enhance ciprofloxacin entry and help explain the synergistic zones observed at some ratios. In contrast, high local concentrations of vanillin or oxidised vitamin C, altered pH, or competition for binding sites may interfere with drug uptake or stability, which would account for the antagonistic patterns seen in other combinations.

In practical terms, vitamin C, gallic acid, and vanillin behaved as weak stand alone antibacterials against multidrug resistant P. aeruginosa in this work, and as modest direct agents against *S. aureus* at only the highest microgram loads. Their main effect was as modifiers of ciprofloxacin activity, and this modification could be in either direction. Many mixtures produced zones similar to or smaller than ciprofloxacin alone, indicating indifferent or mildly antagonistic interactions, while a subset of carefully balanced ratios produced larger zones consistent with synergy. Small gains at the highest gallic acid doses, especially when combined with vitamin C, reached the edge of what can reasonably be detected by disc diffusion, so these findings should be interpreted with caution and confirmed using more quantitative methods. Vanillin enriched preparations tended to shrink the halo size, suggesting that its chemistry or local concentration

was often not favourable for enhancing ciprofloxacin under the current conditions. Future work should therefore consider lower ciprofloxacin loads, freshly prepared vitamin C to minimise oxidation, more precise control of solvent volumes and disc impregnation, and complementary assays such as checkerboard MICs, time kill curves, ethidium bromide accumulation, and biofilm models. Such approaches would help distinguish true helper effects from technical noise and provide a clearer picture of when these food derived compounds act synergistically and when they behave antagonistically with ciprofloxacin.

Future Prospect:

Future work should first repeat and extend these experiments in larger and more diverse collections of multidrug-resistant *S. aureus* and *P. aeruginosa*, including bloodstream, wound, and respiratory isolates, to determine whether the predominantly indifferent and occasionally antagonistic patterns seen here are strain-specific or broadly reproducible. Once this has been established, the experimental framework can be deepened beyond disc diffusion by incorporating minimum inhibitory concentration, checkerboard, time kill, and biofilm assays so that synergistic, additive, and antagonistic interactions are quantified with greater precision and under conditions that more closely resemble clinical infections. Mechanistic studies that monitor efflux activity, membrane permeability, redox balance, and quorum sensing, for example, through fluorescent efflux substrates, membrane probes, and ROS-sensitive dyes, would help connect small changes in inhibition zones to defined cellular processes. In parallel, careful assessment of cytotoxicity in mammalian cell lines, stability of vitamin C and phenolic compounds in realistic vehicles, and simple in vivo infection models will be required before any combination is considered for therapeutic use. Taken together, these steps would allow vitamin C and selected food-derived phenolics to be evaluated not only as potential adjuvants but also as agents that may, under certain conditions, blunt ciprofloxacin activity, thereby providing practical guidance on which combinations can be used safely and which should be avoided during antibiotic therapy.

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