CHARACTERIZATION OF ANTIMICROBIAL PROPERTIES OF *PLEUROTUS OSTREATUS* DERIVED PHENOLIC COMPOUNDS: IMPLICATIONS FOR DRUG DEVELOPMENT AGAINST ANTIBIOTIC-RESISTANT GONOCOCCAL ISOLATES

By

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PREFACE

The research presented in this dissertation/thesis was conducted by the candidate within the Discipline of Biochemistry, School of Life Science, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa. Financial support for this research was provided by the National Research Foundation.

This work has not been submitted in any form to another university, and all results reported are the candidate's own investigations, except where the contributions of others are explicitly acknowledged in the text.

Signed : Yakobi Sinethemba Hopewell

Date: 30 October 2024

As the candidate's supervisor, I have reviewed and approved this thesis for submission.

Supervisor: Dr Ofentse Jacob Pooe

Date: 30 October 2024

DECLARATION 1: PLAGIARISM

I, Sinethemba Hopewell Yakobi, declare that:

1. The research reported in this thesis, except where otherwise indicated or acknowledged, is my original work.
2. This thesis has not been submitted, in full or in part, for any degree or examination to any other university.
3. This thesis does not include data, pictures, graphs, or other information from other individuals, unless specifically acknowledged as sourced from elsewhere.
4. This thesis does not include writing from other individuals, unless specifically acknowledged as sourced from other researchers. Where other written sources are quoted:
    a. Their ideas have been paraphrased, but the general information has been properly referenced.
    b. Exact quotations are enclosed in quotation marks and referenced accordingly.
5. For material used that has been published, I have clearly detailed my role in the work.
6. This thesis primarily consists of material prepared by me, including journal articles and presentations at conferences. Additional material may be included where relevant.

7. This thesis does not include text, graphics, or tables copied from the Internet unless specifically acknowledged, with sources detailed both in the thesis and the References section.

Signed by : Yakobi Sinethemba Hopewell

Date : 30 October 2024

DECLARATION 2: PUBLICATIONS

I, Sinethemba Hopewell Yakobi, hereby declare that the experimental data collection, writing, and analysis for publications listed below were predominantly conducted by Sinethemba Yakobi. Sinethemba Yakobi took a leading role in designing experiments, gathering data, interpreting results, and drafting written materials related to the project. C*o*-authors who assisted in the work were duly acknowledged for their contributions.

Below are the publications that have been produced during the course of this study:

PUBLICATION LIST

PUBLICATION 1:

Title: Antimicrobial Resistance of *Neisseria gonorrhoeae* in Sub-Saharan Populations

Journal: Bacteria (MDPI)

Year: 2022

Status: Published

Authors: Yakobi H. Sinethemba and Pooe J. Ofentse

DOI: 10.3390/bacteria1020009

PUBLICATION 2:

Title: Systematic review of *Neisseria gonorrhoeae* drug resistance development in South Africa

Journal: Brazilian Journal of Microbiology (SPRINGER)

Year: 2024

Status: Published

Authors: Yakobi H. Sinethemba, Magibile B. Yolisa and Pooe J. Ofentse

DOI: 10.1007/s42770-024-01281-6

PUBLICATION 3:

Title: Screening of antimicrobial properties and bioactive compounds of *Pleurotus ostreatus* extracts against *Staphylococcus aureus, Escherichia coli*, and *Neisseria gonorrhoeae*

Journal: Biochemistry Research International (HINDAWI)

Year: 2023

Status: Published

Authors: Yakobi H. Sinethemba, Mkize S. Senzosenkosi and Pooe J. Ofentse

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PUBLICATION 4:

Title: Identification of emerging multidrug-resistant *Neisseria gonorrhoeae* isolates against five major antimicrobial agent options

Journal: Medical Sciences (MDPI)

Year: 2023

Status: Published

Authors: Yakobi H. Sinethemba and Pooe J. Ofentse

DOI: 10.3390/medsci11020028

PUBLICATION 5:

Title: Molecular docking and structure-activity relationship analysis of target compounds against Glyceraldehyde-3-Phosphate Dehydrogenase in azithromycin-resistant *Neisseria gonorrhoeae*

Journal: ChemistrySelect (WILEY)

Year: 2024

Status: Published

Authors: Yakobi H. Sinethemba, Zuma Lindiwe and Pooe J. Ofentse

DOI: 10.1002/slct.202303341

PUBLICATION 6:

Title: Investigation into the interaction between penicillin-resistant and susceptible gonococcal penicillin binding protein-2 and target phenolic ligands through molecular docking studies and structure-activity relationship analysis

Journal: Advances in Pharmacological and Pharmaceutical Sciences (WILEY)

Year: 2024

Status: Published

Authors: Yakobi H. Sinethemba, Zuma Lindiwe and Pooe J. Ofentse

DOI: 10.1155/2024/2585922

PUBLICATION 7:

Title: The molecular and phenotypic analysis of the prevalence and patterns of antimicrobial resistance in *Neisseria gonorrhoeae* isolates from KwaZulu Natal, South Africa

Journal: Scientific Africa (ELSEVIER)

Year: 2024

Status: Published

Authors: Yakobi H. Sinethemba, Nothando Gasa and Pooe J. Ofentse

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PUBLICATION 8:

Title: Antimicrobial potential of organic phenolic compounds from wild mushroom extracts; Impact on proliferation and kinetic growth of multidrug-resistant *Neisseria gonorrhoeae* strains

Journal: Journal of Food Biochemistry (WILEY)

Year: 2024

Status: Published

Authors: Yakobi H. Sinethemba, Lindiwe Zuma, Nothando Gasa and Pooe J. Ofentse

DOI: 10.1155/2024/2336255

PUBLICATION 9:

Title: Gonococcal ophthalmia neonatorum infection transmitted at birth

Journal: American Journal of Biomedical Science and Research

Year: 2022

Status: Published

Authors: Yakobi H. Sinethemba, and Pooe J. Ofentse

DOI: 10.34297/AJBSR.2022.16.002220

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ABSTRACT

The global spread of antimicrobial resistance in *Neisseria gonorrhoeae* presents a major challenge to treating gonorrhoeal infections, necessitating immediate intervention strategies. This study investigates the prevalence and mechanisms of antibiotic resistance in *N. gonorrhoeae* isolates from KwaZulu Natal, South Africa, where antimicrobial resistance monitoring is limited. Utilizing molecular and culture methods, significant resistance levels were detected: 48% of isolates were fully resistant to ciprofloxacin, 14% to both penicillin and tetracycline, and one isolate showed resistance to azithromycin, while ceftriaxone remained universally effective. The extracts exhibited varying antimicrobial activities against *E. coli* and *S. aureus,* with a mean extract concentration of $1x10^{-5}$ mg/mL. In contrast, *N. gonorrhoeae* isolates showed a mean MIC concentration of $1x10^{-3}$ mg/mL, indicating a higher resistance to the target extracts compared to *E. coli* and *S. aureus*. The high resistance to penicillin, with MICs exceeding 32 µg/mL, is attributed to the presence of penicillinase-producing plasmids and mutations in penicillin-binding proteins (PBPs), particularly in the *penA* gene. Logistic regression indicated a strong correlation between bacterial growth rates and tetracycline concentrations, emphasizing the complexity of resistance dynamics. The study also explored flavonoid compounds as alternative therapies, with quercetin showing notable antibacterial properties. Molecular docking and dynamics simulations were employed to examine quercetin's interaction with critical bacterial proteins, penicillin-binding protein 2 (PBP2) and

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), essential for cell wall synthesis and metabolism. Quercetin demonstrated strong binding affinity to the active sites of these proteins, potentially inhibiting their function. Molecular dynamics (MD) simulations provided insights into the long-term stability and dynamic behaviour of the quercetin-protein complexes, showing consistent and secure binding within the active sites of PBP2 and GAPDH. Analysis of root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF) confirmed the stability of these protein structures during simulation. Binding-free energy calculations using the molecular mechanics method confirmed quercetin's strong inhibitory potential against both PBP2 and GAPDH. Structural insights from Procheck and other computational tools identified crucial residues in the binding pockets, providing valuable information for developing more effective quercetin derivatives or other flavonoid-based therapeutics. This comprehensive study underscores quercetin as a promising alternative treatment for drug-resistant *N. gonorrhoeae* and emphasizes the need for continued exploration of flavonoid compounds in combating antimicrobial resistance. The findings advocate for a global collaborative effort to combat antimicrobial resistance in *N. gonorrhoeae*, involving robust surveillance systems, advanced molecular techniques, and novel antimicrobial agents to curb the spread of resistant strains, ensure effective treatment, and safeguard public health.

DEDICATION

This thesis is dedicated to my beloved family, without whom this academic journey would not have been possible, your unwavering support and understanding have been my rock and anchor throughout this endeavour. To my precious daughter and only son, Eliana and Alitha Yakobi, your presence and inspiration have filled my life with purpose and joy, I live for you. Thank you guys for being the cornerstone of my academic and personal life.

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Now unto Him that is able to keep us from falling, and to present us faultless before the presence of His glory with exceeding joy, **to the only wise God the Saviour**, be glory and majesty, dominion and power, both now and ever. Amen.

I wish to express my deepest thanks to the following people:

Dr O.J. Pooe, you consistently demonstrated a willingness to share your expertise with others. I would like to extend my sincere appreciation to you for accepting me as a student and for demonstrating your care, guidance, commitment, and assistance throughout the duration of the study project. I am deeply grateful for the tremendous dedication and commitment you have demonstrated in ensuring the accomplishment of this research endeavour.

Senzosenkosi Mkhize, Lindi Zuma, Yolisa Magibile and Nothando Gaza, students currently conducting research in the laboratory of Dr O.J. Pooe, I would like to extend my sincere appreciation for the assistance and support you have provided.

CHAPTER 1: INTRODUCTION

Antimicrobial susceptibility profiles and worldwide trends of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) present a significant prevalence of penicillin-, tetracycline-, and ciprofloxacin- resistance, obviating the use of the respective drug in empiric treatment recommendations (Unemo et al., 2019; Unemo & Shafer, 2014; Whittles et al., 2018). Rising azithromycin resistance rates have also been documented globally, which is concerning given that azithromycin is still part of the current recommended combination treatment for gonococcal infections (Chen et al., 2019). Along with rising moderate- level azithromycin resistance, there have been reports of high-level azithromycin resistance with a Minimum Inhibitory Concentration (MIC) greater than 256 mg/L (Yan et al., 2019). The emergence of multidrug resistant (MDR) *N. gonorrhoeae* strains have been well documented by surveillance programmes all around the world. These strains present a significant treatment challenge and to overlap the limitations of the available antimicrobial drugs on these strains, novel drugs with new mechanisms of action are synthesised (Jacobsson et al., 2018; Yang & Yan, 2020).

There has been a growing body of research on phenolic compounds found in various mushroom species, which have demonstrated strong antibacterial properties in recent years (Ecevit et al., 2022; Miklasińska-Majdanik et al., 2018). These compounds have been shown to have antiviral, anticancer, antibiotic and

antibacterial properties. Although the mechanisms of antibacterial activity of these phenolic compounds is not completely understood, it has been suggested that these compounds involve several sites of action at the cellular level (Abdel-Aty et al., 2022; Miklasińska-Majdanik et al., 2018). Several researchers have provided explanations for this activity, citing factors such as the alteration of cell membrane permeability, the impact of phenolic compounds binding to enzymes and affecting intracellular processes, and the potential changes in cell wall rigidity due to interactions with the cell membrane (Abdel-Aty et al., 2022; Ecevit et al., 2022; Miklasińska-Majdanik et al., 2018). Thus, enhancing the lipophilicity of phenolic compounds enhances their ability to combat bacteria by enabling easier interaction with the cell membrane. It was also suggested that this could potentially result in long-term damage to the cytoplasmic membrane and cause the coagulation of cell content, potentially hindering the function of intracellular enzymes. Research has shown that condensed phenylpropanoids, such as tannins, can interfere with the integrity of cell membranes and hinder metabolic processes by binding to enzymes. Similarly, phenolic acids have been found to disrupt cell membrane integrity and lead to the leakage of vital intracellular components (Donadio et al., 2021; Kumar & Pandey, 2013). Flavonoids are hydroxylated phenolic compounds that are produced by plants in response to microbial infection. The chemical constitution of flavonoid compounds is determined by their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization (Panche et al., 2016, Perry et

al.,2023). A recent study showed that flavonoids may interact with soluble proteins found outside bacterial cell walls, facilitating the creation of complexes. It was further noted that flavonoids may also potentially impact protein and RNA production by decreasing both energy consumption and DNA synthesis (Roy et al., 2022; Tungmunnithum et al., 2018). Flavonoids are a noteworthy group of polyphenolic compounds with a benzo-pyrone structure that are found in abundance in plants. These compounds are synthesised via the phenylpropanoid pathway. There is a wide range of pharmacological activities associated with secondary metabolites of phenolic nature, specifically flavonoids, based on the existing evidence (Panche et al., 2016). The potential health benefits resulting from the antioxidant properties of these polyphenolic compounds have sparked a recent interest in these substances (Singh et al., 2023). Functional hydroxyl groups in flavonoids play a crucial role in their antioxidant effects, as they scavenge free radicals and chelate metal ions. Understanding the process of chelation is crucial for protecting important biomolecules from damage caused by radical generation (Makhoba et al., 2020; Panche et al., 2016). The investigation into the antimicrobial potential of *Pleurotus ostreatus* extracts against drug resistant *N. gonorrhoeae* is driven by several critical and multifaceted considerations. Firstly, the economic burden imposed by rising drug-resistant *N. gonorrhoeae* is substantial, necessitating more costly and prolonged treatments and leading to greater healthcare expenditures. Developing cost-effective alternatives from natural sources like *P. ostreatus* could mitigate

these financial pressures. Furthermore, access to effective antibiotics is often limited in low- and middle-income countries, particularly the Sub-Saharan Africa. *P. ostreatus*, being widely cultivated and inexpensive, presents an accessible option for antimicrobial agents, thus promoting global health equity by improving treatment availability and efficacy across diverse regions. Utilizing natural compounds from mushrooms offers a more sustainable approach, reducing reliance on synthetic antibiotics and their ecological impact. The phenolic compounds in *P. ostreatus* potentially offer diverse and unique mechanisms of action against bacteria, which may lower the risk of resistance development compared to single-target synthetic antibiotics. Additionally, these compounds might exhibit synergistic effects with existing antibiotics, enhancing their effectiveness and allowing for reduced dosages, thus mitigating side effects and resistance evolution. Lastly, the growing consumer preference for natural and plant-based healthcare products aligns with the exploration of *P. ostreatus* extracts. Establishing their efficacy could increase acceptance and integration of natural antimicrobial treatments within mainstream medical practice. In this context, the aim of this study was to determine the antibacterial activity of the phenolic compounds (phenolic acids and flavonoids) of *P. ostreatus* against drug-resistant *N. gonorrhoeae*.

1.1 AIMS

The study aims was to identify antimicrobial compounds derived from *Pleurotus ostreatus*, assess their effectiveness against a variety of multidrug-resistant *Neisseria gonorrhoeae* isolates, and explore the mechanisms by which these compounds interact with the proteins of the microorganism.

1.2 OBJECTIVES OF THE STUDY

1.2.1 Assess Antibiotic Resistance Profiles

1.2.1.1 Evaluate the prevalence, trends and minimum inhibitory concentrations (MICs) values of resistance to various antibiotics, including ciprofloxacin, penicillin, tetracycline, azithromycin, and ceftriaxone, in *Neisseria gonorrhoeae* isolates from KwaZulu Natal, South Africa.

1.2.2 Identify Genetic Mechanisms of Resistance

1.2.2.1 Utilize genomic analyses to pinpoint specific mutations within penicillin-binding proteins (PBPs) and other genetic elements implicated in antibiotic resistance mechanisms in *Neisseria gonorrhoeae*.

1.2.2.2 Employ PCR and molecular based studies to validate a concise set of genetic loci associated with susceptibility to penicillin and tetracycline. Investigate the potential correlation between mutational profiles observed in the *gyrA* and *parC* genes and the resultant resistance levels to ciprofloxacin. Additionally, scrutinize the prevalent patterns of resistance to azithromycin and ceftriaxone in target bacterial strains.

1.2.3 Explore Alternative Therapeutic Agents

1.2.3.1 Investigate the impact of the extraction process of *Pleurotus ostreatus* on various parameters including yield, productivity, bioactive compound content, and antibacterial activity against clinical isolates of *Neisseria gonorrhoeae*.

1.2.3.2 Evaluate the antibacterial properties and efficacy of individual phenolic compounds against microorganisms that have developed resistance to conventional antibiotics. Additionally, assess the modulatory effects of selected phenolic compounds on the proliferation of target clinical isolates of *N. gonorrhoeae* exhibiting a diverse range of antimicrobial resistance.

1.2.4 Perform Molecular Docking Studies

1.2.4.1 Employ molecular docking simulations to evaluate the binding affinity and interaction mechanisms of phenolic compounds with essential bacterial proteins. Specifically, investigate the interactions of these compounds with key proteins involved in cell wall synthesis, penicillin-binding protein 2 (PBP2), and enzymes central to the glycolytic pathway, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

1.2.5 Conduct Molecular Dynamics Simulations

1.2.5.1 Utilize molecular dynamics simulations to analyse the stability and dynamics of ligand-protein complexes exhibiting the highest binding

affinity. Investigate the interaction mechanisms between phenolic compounds and target drug-resistant bacterial cells, to provide insights into the potential inhibitory effects of these compounds on the targeted bacteria, elucidating their mechanisms of action and therapeutic potential.

1.3 OUTLINE OF DISSERTATION/THESIS STRUCTURE

1.3.1 The thesis is divided into 10 different chapters as below.

1.3.1.1 **Chapter 1**: **Introduction** (this chapter contains introduction, aims, and objectives as well as the thesis organization.)

1.3.1.2 **Chapter 2**: **Literature Review** (this chapter contains the literature review, background, an overview of antimicrobial resistance mechanisms in *N. gonorrhoeae*, current diagnostic methods and surveillance efforts, existing treatments and challenges in combating resistance, research rationale and scope)

1.3.1.3 **Chapter 3**: **Materials and Methods** (this chapter contains methods used in the characterization of *N. gonorrhoeae* isolates, antibiotic susceptibility testing methodologies, genomic analysis techniques, extraction and analysis of *Pleurotus ostreatus* bioactive compounds and molecular docking and dynamics simulations)

1.3.1.4 **Chapter 4**: **Identification of emerging multidrug-resistant *Neisseria gonorrhoeae* isolates against five major antimicrobial agent options** (this chapter contains article reporting on the biochemical

detection and analysis of multidrug-resistant strains of *Neisseria gonorrhoeae* isolates taken in KwaZulu Natal that were used in this project).

1.3.1.5 **Chapter 5**: **A molecular and phenotypic analysis of the prevalence and patterns of antimicrobial resistance in *Neisseria gonorrhoeae* isolates from KwaZulu natal, South Africa** (in this chapter the molecular resistance patterns and identified gaps in strategies for effective management and control of gonorrhoeae was investigated).

1.3.1.6 **Chapter 6**: **Screening of antimicrobial properties and bioactive compounds of *Pleurotus ostreatus* extracts against *Staphylococcus aureus*, *Escherichia coli*, and *Neisseria gonorrhoeae*** (this chapter reports on the antibacterial properties of crude *Pleurotus ostreatus* extracts against a range of target bacterial strains).

1.3.1.7 **Chapter 7**: A**ntimicrobial potential of organic phenolic compounds from mushroom extracts: impact on proliferation and kinetic growth of multi-drug resistant *Neisseria gonorrhoeae* strains** (this chapter investigates the bioactivity impact of these compounds on the proliferation and kinetic growth of multi-drug resistant strains of *Neisseria gonorrhoeae*).

1.3.1.8 **Chapter 8: Investigation into the interaction between penicillin-resistant and susceptible gonococcal penicillin binding protein-2 and target phenolic ligands through molecular docking studies and**

structure-activity relationship analysis (this chapter reports on the structural and functional analysis, providing insights the potential as inhibitory interactions using five target phenolic compounds, previously identified for their antimicrobial properties, against susceptible and resistant PBP2).

1.3.1.9 **Chapter 9: Molecular docking and structure-activity relationship analysis of target compounds against glyceraldehyde-3-phosphate dehydrogenase in azithromycin-resistant *Neisseria gonorrhoeae*** (this chapter focuses on the crystallographic conformation of GAPDH, a key enzyme in glycolysis, derived from *Neisseria gonorrhoeae* strain NCCP11945)

1.3.1.10 **Chapter 10: Conclusion and Future Studies** (this section contains the interpretation of results in the context of research objectives, the implications of findings for antimicrobial resistance management and the limitations of the study and areas for future research), **Conclusion** (this section contains a summary of key findings and their significance and contributions to the field and recommendations for practice), **Appendices** (this section contains conference outputs and a list of publications from this study)

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CHAPTER 2: LITERATURE REVIEW

2.1 BACKGROUND

Neisseria gonorrhoeae is a gram-negative diplococcus beta proteobacterium that is responsible for causing gonorrhoeae, a highly common sexually transmitted infection (STI) that is found all over the world (Unemo, 2015a; Unemo et al., 2019; Whittles et al., 2018). While *N. gonorrhoeae* infections are not typically life-threatening, the disease is widespread, especially among males. Conversely, women are more prone to experiencing complications during infection, such as the development of pelvic inflammatory diseases (Unemo, 2015a; World Health Organization, 2016). Urogenital infections are typically without symptoms, and it is common for over 50% of females to experience at least one urogenital infection during their lifetime (Biggel et al., 2019). The bacterium *N. gonorrhoeae* can lead to complications in the female upper genital tract and, less commonly, epididymitis in males. These infections can result in reproductive challenges and potential infertility for individuals of all genders. Individuals at a higher risk for *N. gonorrhoeae* infection are those who are part of sexual networks that practice unprotected sex with multiple partners, individuals engaged in commercial sex work, men who have sex with men, and young heterosexual individuals (Chesson et al., 2017; Kirkcaldy, 2019; Kularatne et al., 2018; Mabonga et al., 2019; Tsevat et al., 2017). There has been a significant rise of 19% in new cases of gonococcal infections globally in recent times and moreover, based on data from the WHO, there has been a steady rise in reported *N.*

gonorrhoeae infections among females aged 15–49 in Sub-Saharan Africa, with an annual increase of 2.6–5.0%. Further modelling research indicates that Sub-Saharan Africa stands out as the sole region experiencing substantial incidences of STIs (Kirkcaldy, 2019; Maduna et al., 2020; Workneh et al., 2020). There is a significant amount of research indicating that there are differences in infection susceptibility between males and females. One key factor is the variation in sensitivity to infection in the male and female genital tracts. Additionally, studies have shown that transmission from males to females tends to be more successful. These findings have been widely reported in the scientific community. Additionally, many infections in females go unnoticed as they show no symptoms and are left untreated (Kularatne et al., 2018; Mabonga et al., 2019; World Health Organization, 2016).

Undiagnosed infections in women can have serious consequences, such as pelvic inflammatory disease, which may result in ectopic pregnancy, tubal infertility, or ongoing pelvic discomfort (Forero et al., 2018; Kularatne et al., 2018; Mathebula et al., 2020). In addition, some sexually transmitted infections (STIs) can increase the risk of acquiring and transmitting the human immunodeficiency virus (HIV). Therefore, recognising and addressing sexually transmitted infections (STIs) and other infections affecting the reproductive tract are crucial aspects of sexual and reproductive health services (Guvenc et al., 2020; Torrone et al., 2018).

Health services in several African countries offer STI management, but there is a worrying increase in new and untreated cases of *N. gonorrhoeae* infections. This is mainly because of treatment failures and the fact that the disease can often show no symptoms. Given its capacity to induce infections without displaying symptoms, coupled with its frequent alterations in surface antigens and resistance to antibiotics, *N. gonorrhoeae* continues to pose a substantial global health concern (Lovett & Duncan, 2019; Springer & Salen, 2021). In low-resource settings where microbiological STI identification is not available, symptom-based management is commonly used (Quillin & Seifert, 2018). It is common for patients suspected of *N. gonorrhoeae* infection to be prescribed drug-combination treatment without undergoing diagnostic tests, even though this approach has drawbacks like the potential development of antimicrobial resistance (Glasgow, 2020). Effective genetic resistance mechanisms have been developed by *N. gonorrhoeae*, leading to a significant increase in antimicrobial resistance worldwide. There are different mechanisms through which resistance can be acquired, such as through the chromosome, plasmids, and efflux pumps that are resistant to multiple drugs (Kirkcaldy, 2019; Springer & Salen, 2021). Improper use of treatment drugs plays a major role in the development of antibiotic resistance (St. Cyr et al., 2020). At present, the primary treatments for urinary tract and respiratory infections consist of antibiotics like amoxicillin, sulfamethoxazole, or trimethoprim (Fourie et al., 2021). On the other hand, bloodstream infections are managed by employing combinations of antibiotics

such as ampicillin and gentamicin or ceftriaxone (Maduna et al., 2020; Tadesse et al., 2017). Recent research findings have brought to light a concerning level of resistance to commonly used antibiotics in Sub-Saharan countries when it comes to *N. gonorrhoeae* (Tadesse et al., 2017). This emphasises the pressing need to thoroughly evaluate levels of antibiotic resistance and fill in any gaps in the knowledge. It is of utmost importance to address the challenge of drug-resistant strains of *N. gonorrhoeae* by developing innovative treatment therapies and vaccines, as the existing options are quite limited (Unemo & Workowski, 2018; Xu et al., 2018). It is crucial to prioritise the development of innovative treatments that target the constant structures of host cells that gonococci encounter during adhesion, colonisation, and invasion. This approach is vital for effectively managing the disease. When surveillance was carried out in the Sub-Sahara, the recruitment process often resulted in a low number of participants (Yakobi & Pooe, 2022). This trend is believed to be influenced by various factors, including men experiencing urethral discharge seeking medical assistance from private doctors or resorting to purchasing medication from street vendors. In addition, there can be difficulties when it comes to transporting samples to processing laboratories and delays that may occur when laboratory staff process isolates. Therefore, it is important to exercise caution when interpreting data from the Sub-Saharan region due to the limited sample sizes, even though there may be an increase in global antimicrobial resistance levels of *N. gonorrhoeae* (Goire et al., 2011; Perovic & Schultsz, 2016; Workneh et al., 2020; Yakobi & Pooe, 2022).

There is an urgent requirement for improved surveillance and monitoring of *N. gonorrhoeae* in sub-Saharan populations, with a comprehensive approach being essential to meeting the goals of the WHO global health sector plan. The region demonstrates a noteworthy level of antibiotic resistance across different antibiotics, emphasising the significance of accurate microbiological identification and susceptibility testing. It is absolutely essential for the global health community to come together and support these countries in tackling the identified concerns and preventing the emergence of public health risks related to antimicrobial resistance. Considering the lack of a reliable preventive measure for *N. gonorrhoeae* infections, it is crucial to prioritise the development of innovative strategies and broaden the range of potential drug candidates. It is crucial to continuously conduct research and development to address the challenge of antimicrobial resistance and improve public health outcomes.

2.2 ANTIMICROBIAL RESISTANCE OF *N. GONORRHOEAE* IN AFRICA

In 2016, the World Health Organisation (WHO) released a report on the global rate of new *N. gonorrhoeae* infections. The findings revealed that a significant number of individuals, totalling over 75 million people, are exposed to this infection each year. The report highlighted that females experienced a rate of 19 new infections per 1000 individuals, while males had a slightly higher rate of 24 per 1000 individuals (Kularatne et al., 2018; World Health Organization, 2016).

Gonococcal infections are most prevalent in Africa. Teens and young adults are particularly affected by the association between *N. gonorrhoeae* infection and the risk of HIV type 1. Findings from 102 studies conducted between 2005 and 2014 in Sub-Saharan Africa, which included countries with moderate-t*o*-high HIV prevalence, showed notable disparities in the number of studies conducted in different countries, see Figure 2.1 (Dubbink et al., 2018; Guvenc et al., 2020; Kharsany & Karim, 2016). The majority of studies were conducted in South Africa, Tanzania, and Kenya. Internationally, a comprehensive study analysed data from 147 prevalence studies in 56 countries. The study found that the overall global prevalence of current gonorrhoeae infection is estimated to be 2.2%. Notably, Africa had the highest incidence rate at 5.0%. However, the studies mentioned only cover a small portion of the sub-Saharan population (Kassa et al., 2020). Many countries in the region do not have effective methods for collecting data on sexually transmitted infections (STIs) and lack national surveillance systems. The syndromic approach to STI diagnosis and management, which does not involve routine collection of genital specimens for laboratory analysis, is widely accepted in African countries. However, there have been noticeable advantages to the syndromic approach, as it has led to a decrease in bacterial sexually transmitted infections over the years (Mabonga et al., 2019; Tadesse et al., 2017).

After receiving reports of antimicrobial resistance to quinolones in *N. gonorrhoeae,* the World Health Organisation (WHO) has advised the need for regular and thorough monitoring of this resistance (Unemo et al., 2021). It is crucial to improve surveillance efforts in order to monitor resistance patterns and effectively guide treatment strategies. An international workshop was held in Harare, Zimbabwe, to assess the advancements made by Sub-Saharan countries in establishing national surveillance systems for monitoring antimicrobial resistance in *N. gonorrhoeae* (Wi et al., 2017). There were a total of 11 countries that took part in this event. These countries include Benin, Cameroon, Ethiopia, Ghana, Kenya, Madagascar, Nigeria, South Africa, Tanzania, Uganda, and Zimbabwe. Despite the slow response, there have been several studies conducted in certain African countries to monitor antimicrobial resistance in *N. gonorrhoeae*, with others ready to be implemented (Wi et al., 2017). During a study conducted in Cameroon from 2012 to 2018, a total of 449 strains of *N. gonorrhoeae* were isolated. It was found that a considerable number of these strains exhibited resistance to ciprofloxacin, benzylpenicillin, and tetracycline (Crucitti et al., 2020). In the Central African Republic, the most recent study on *N. gonorrhoeae* antimicrobial resistance was conducted in 1984 for males and 1999 for females. A study conducted in Zambia examined the relationship between various sociodemographic and clinical risk factors and the prevalence of Chlamydia trachomatis and *N. gonorrhoeae*. The findings indicated that there

were associations with younger age, lower education, and other relevant factors (Meheus et al., 1984).

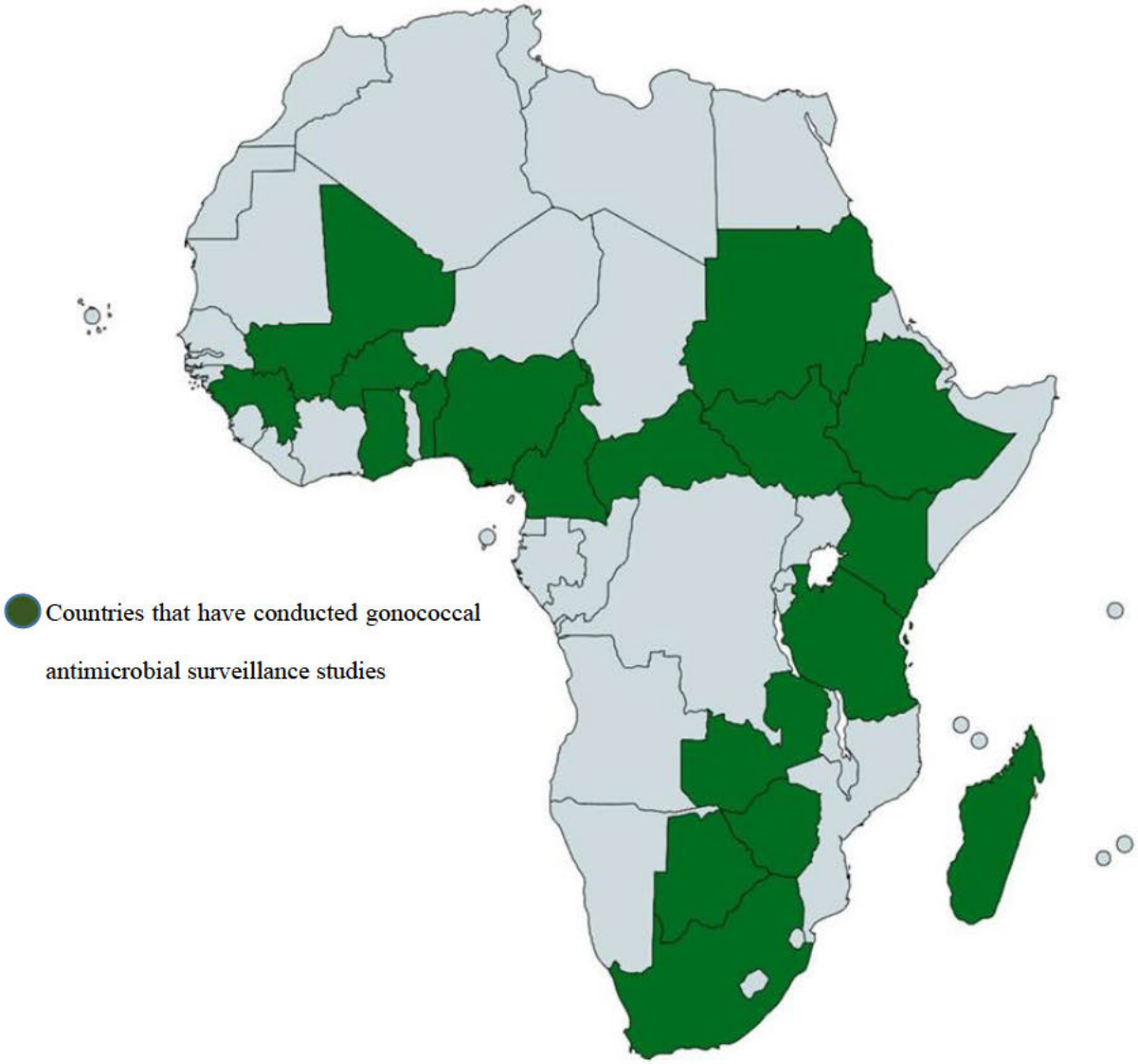

Figure 2.1: Sub-Saharan African nations with reported surveillance studies on gonococcal infections.

In Sudan, a study was conducted between January and October 1999, which discovered the presence of *N. gonorrhoeae* in 2% of pregnant women who visited the Khartoum Teaching Hospital Antenatal Clinic. This finding highlights the

importance of understanding and addressing the prevalence of this infection among pregnant women (Abdelrahim et al., 2017; Connolly et al., 2020; Ortashi et al., 2009). Similarly, in Botswana, a study conducted on prenatal care attendees found a 3% prevalence of *N. gonorrhoeae* (Romoren et al., 2007). A recent surveillance study in South Africa revealed a concerning prevalence of ciprofloxacin resistance. However, there is some positive news, as effective cephalosporins were found to be working well. It is worth noting that there were a few isolated cases of cefpodoxime resistance reported in Gauteng. In Johannesburg, South Africa, a significant proportion of *N. gonorrhoeae* isolates from immunocompromised males displayed resistance to multiple drugs, including ciprofloxacin and azithromycin. In KwaZulu-Natal province, a significant number of *N. gonorrhoeae* strains showed resistance to azithromycin, with a high percentage of them being multidrug-resistant. There is a clear need for a thorough evaluation of azithromycin resistance across the country, as disparities in surveillance data have brought this issue to light. After analysing the latest research papers on *N. gonorrhoeae* infections in Africa, it was found that a total of 15,546 individuals were studied. Out of these, 7.9% were found to be primarily infected with *N. gonorrhoeae* (Kularatne et al., 2018; Maduna et al., 2020; Peters & Maduna, 2020; Wynn et al., 2018). There was a higher occurrence of infections in the 21–25 age group, with men showing a greater frequency than women in Nigeria, Ethiopia, and Ghana, see Table 2.1 and Figure 2.2.

The provided data on the prevalence of *N. gonorrhoeae* infections in 15 Sub-Saharan countries (Table 2.1 and Figure 2.2) presents several limitations that need to be acknowledged for a comprehensive understanding of its scientific validity and applicability. There are significantly more female participants (8259) compared to male participants (7287). This gender imbalance can introduce bias in the prevalence rates. Infections might be underreported in males, skewing the data and affecting the reliability of comparative analysis between genders. The number of participants with positive gonococcal infection is disproportionately higher in some countries (Ghana, South Africa) compared to others. This could indicate differences in diagnostic capacities, healthcare access, or reporting practices across countries, affecting the consistency and comparability of data. Some countries have zero male participants (Benin, Burkina Faso, Guinea, Mali, Sudan, Zambia, and Zimbabwe), and the absence of male participants in these countries can lead to incomplete epidemiological insights and potentially misrepresent the true prevalence of *N. gonorrhoeae* in these populations. Countries like Central African Republic and Mali have very small sample sizes for both genders. Small sample sizes can result in higher variability and lower reliability of prevalence estimates. The data might not be representative of the broader population in these countries. There is no information on whether standardized methodologies were used across different countries. Differences in diagnostic criteria, sample collection methods, and laboratory procedures can lead to inconsistent data, complicating cross-country comparisons and

aggregations. Socio-cultural factors affecting participation rates and healthcare-seeking behaviour are not addressed. Stigma, gender norms, and cultural attitudes towards sexual health can influence who participates in studies and how honestly, they report infections, introducing biases in the data. The data represents only 15 countries within Sub-Saharan Africa. This regional limitation means the findings cannot be generalized to the entire Sub-Saharan region or other parts of Africa, potentially missing variations in prevalence and epidemiological trends.

While the data provides a snapshot of *N. gonorrhoeae* prevalence in select Sub-Saharan countries, these limitations highlight the need for caution in interpretation. Future studies should aim for balanced gender representation, larger and more representative sample sizes, standardized methodologies, and consideration of socio-cultural and confounding factors to enhance the reliability and applicability of the findings.

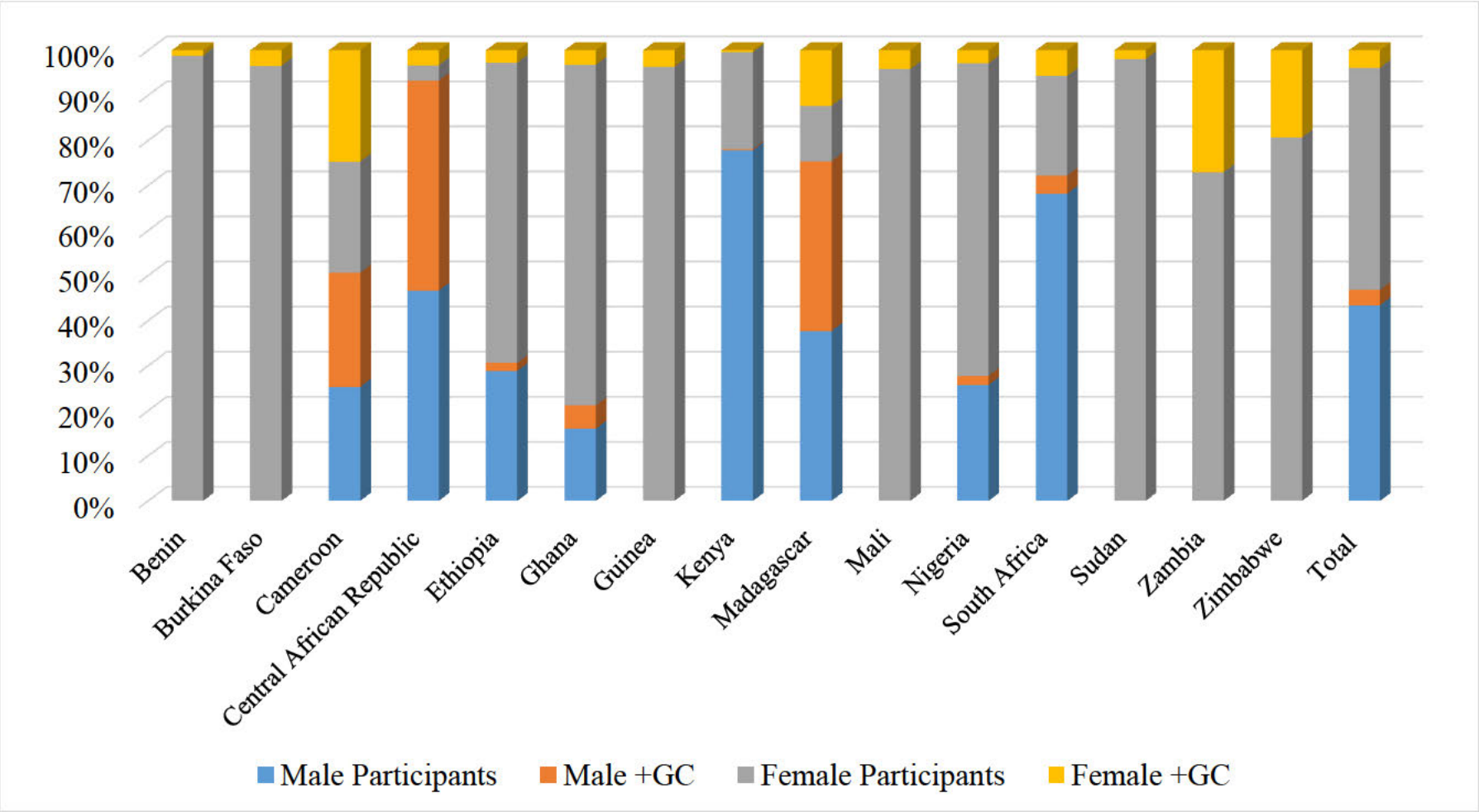

Figure 2.2: Prevalence of *N. Gonorrhoeae* infections in 15 Sub-Saharan countries by gender and gonococcal infection as listed in Table 2.1.

Table 2.1: The prevalence of *N. Gonorrhoeae* infections in 15 Sub-Saharan African countries: gender distribution and gonococcal infections. *(+GC – *N. gonorrhoeae* positive identification)

Country	**Sample Size**	**Male Participants**	**Male +GC**	**Female Participants**	**Female +GC**
Benin	81	0	0	81	1
Burkina Faso	367	0	0	367	13
Cameroon	79	40	40	39	39
Central African Republic	30	28	28	2	2
Ethiopia	907	274	17	633	26
Ghana	3079	539	173	2540	108
Guinea	237	0	0	237	9
Kenya	3696	2895	9	801	15
Madagascar	126	95	95	31	31
Mali	114	0	0	114	5
Nigeria	2868	777	61	2091	87
South Africa	3495	2639	156	856	220
Sudan	151	0	0	151	3
Zambia	116	0	0	116	43
Zimbabwe	200	0	0	200	48
Total	15546	7287	579	8259	650

2.3 THE EVOLUTION OF ANTIMICROBIAL RESISTANCE

2.3.1 Sulphonamides

In the 1930s, sulphonamides were first used to treat *N. gonorrhoeae* infections. However, in 1944, the rates of treatment failure among World War II soldiers in the Italian army reached a staggering 75%. Antimicrobials focus on dihydropteroate synthetase (DHPS), an essential enzyme in folic acid production (Costa-Lourenço et al., 2017a). They compete with the usual substrate, *p*-aminobenzoic acid. Resistance mechanisms include an increase in *p*-aminobenzoic acid production or the presence of mutant DHPS with a lower affinity for antibiotics (Munita & Arias, 2016). During the 1960s, a new approach called combination therapy with trimethoprim was introduced to improve effectiveness. Trimethoprim hinders the transformation of dihydrofolate to tetrahydrofolate through the action of dihydrofolate reductase (DHFR) (Tadesse et al., 2017). However, genetic alterations in *N. gonorrhoeae* DHFR can occur, leading to decreased vulnerability to trimethoprim (Fourie et al., 2021). Until the 1970s, a combination therapy approach was employed to target bacterial folic acid synthesis by inhibiting DHPS. Resistance mechanisms involve the increased production of *p*-aminobenzoic acid or alterations in the *folP* gene that codes for DHPS, leading to a decrease in the effectiveness of antimicrobials (Costa-Lourenço et al., 2017a; Springer & Salen, 2021), see Table 2.2 and Figure 2.3.

2.4 ANTIMICROBIAL POTENTIAL OF ORGANIC PHENOLIC COMPOUNDS FROM MUSHROOM EXTRACTS

As previously emphasised, antimicrobial resistance in *Neisseria gonorrhoeae* (*N. gonorrhoeae*) is a major global challenge. Resistance to penicillin, tetracycline, ciprofloxacin, and azithromycin is widespread and has serious implications. The rise of MDR strains adds another layer of complexity to treatment efforts, underscoring the pressing demand for innovative antimicrobial agents. Phenolic compounds derived from different mushroom species have gained recent attention due to their remarkable antibacterial activity, providing potential solutions to address the issue of antibiotic-resistant bacteria. These phenolic compounds have been found to possess antiviral, anticancer, and antibiotic properties (dos Reis et al., 2022; Fadiji & Babalola, 2020; J. V. Pham et al., 2019). They exert their antibacterial effects by interacting with cells at a cellular level. Although the precise mechanisms remain unclear, researchers speculate that phenolic compounds alter the permeability of cell membranes, hinder intracellular processes by binding to enzymes, and compromise the integrity of cell walls. Enhancing the lipophilicity of phenolic compounds can boost their antibacterial activity by promoting stronger interactions with bacterial cell membranes, which may lead to irreversible damage (Cui et al., 2021; Fakoya et al., 2020). Phenylpropanoids that are condensed, like tannins, have the ability to disrupt the integrity of membranes and enzyme activity. On the other hand, phenolic acids can compromise the integrity of membranes, leading to leakage of

intracellular contents (Jacobsson et al., 2018). Flavonoids, a significant group of polyphenolic compounds found in plants, have been observed to interact with soluble proteins outside bacterial cell walls. This interaction has the potential to form complexes and affect protein and RNA production, leading to the inhibition of bacterial growth (Panche et al., 2016).

Flavonoids possess functional hydroxyl groups that give them antioxidant properties, making them highly promising in the fight against antimicrobial resistance. Through the process of scavenging free radicals and chelating metal ions, flavonoids have the ability to safeguard biomolecules from oxidative damage (Donadio et al., 2021; Roy et al., 2022; Sarian et al., 2017). This not only provides antimicrobial effects but also presents potential therapeutic advantages. In light of recent findings, the identification of antibacterial properties in phenolic compounds derived from mushrooms has opened up a realm of possibilities of combating multidrug-resistant bacterial pathogens such as *N. gonorrhoeae*. This discovery holds great promise for the development of alternative treatments in the future. Investigation is necessary to fully understand how these compounds work and their potential for treating antimicrobial resistance.

2.4.1 Penicillin

Penicillin is a widely used antibiotic that has revolutionised the field of medicine. It has been instrumental in treating various bacterial infections and has saved countless lives. The discovery of penicillin by Alexander Fleming in 1928

marked a significant milestone in the history of microbiology and science. In 1943, penicillin became a successful treatment for *N. gonorrhoeae* after previous attempts with sulphonamide had failed. It hinders the synthesis of bacterial cell walls by attaching to penicillin-binding proteins (PBPs) in the periplasmic space (Costa-Lourenço et al., 2017a; Młynarczyk-Bonikowska et al., 2019). Resistance mechanisms involve alterations in genes related to cell wall production or structures that impact drug concentration in the periplasm. In the 1960s, there was a rise in reduced penicillin susceptibility, and by the 1970s, the MICs reached as high as 128 g/mL. Plasmid-mediated β-lactamase gene types, particularly blaTEM, have played a significant role in the emergence of penicillinase-producing *N. gonorrhoeae* (PPNG), marking the end of the penicillin era (Rambaran et al., 2019), see Table 2.2 and Figure 2.3.

2.4.2 Tetracycline

In the 1950s, tetracycline emerged as a viable treatment option for individuals with penicillin allergies. The overexpression of certain genes hindered the effectiveness of tetracycline, resulting in the development of tetracycline-resistant *N. gonorrhoeae*. In 1985, a significant level of resistance developed as a result of the expression of the *tetM* protein. Tetracyclines hinder the binding of aminoacyl-tRNA to the mRNA-ribosome complex, mainly by attaching to the 30S ribosomal subunit, resulting in a decrease in protein synthesis (Costa-Lourenço et al., 2017a; Elkashif & Seleem, 2020). Chromosomal mutations that

impact ribosomal protein structure have been found to increase efflux and decrease the influx of tetracycline, resulting in the development of resistance (Munita & Arias, 2016), see Table 2.2 and Figure 2.3.

2.4.3 Quinolone

In 1983, Ciprofloxacin was introduced as a single-dose treatment for *N. gonorrhoeae* at a dosage of 250 mg (Costa-Lourenço et al., 2017a). In the 1930s, sulphonamides were first used to treat *N. gonorrhoeae* infections. However, the CDC later advised taking a single dose of 500 mg. Despite the early identification of reduced susceptibility and therapeutic failures, ciprofloxacin remained a widely used treatment for an additional 10–25 years worldwide (D'Atanasio et al., 2020a). Quinolones specifically focus on DNA gyrase and topoisomerase IV, which play a crucial role in various DNA processes (D'Atanasio et al., 2020a). Resistance mechanisms include an increase in *p*-aminobenzoic acid production or the presence of mutant DHPS with a lower affinity for antibiotics. Resistance can develop due to mutations in the quinolone resistance-determining region (QRDR), located near the DNA binding site of these enzymes (Costa-Lourenço et al., 2017a), see Table 2.2 and Figure 2.3.

Table 2.2: A summary of the molecular determinants and resistance mechanisms of *N. gonorrhoeae*.

Antimicrobial class	**Resistance determinants/mechanisms**
Sulphonamide	Excessive production of *p*-aminobenzoic acid, resulting in the dilution of the sulphonamide. When there is a mutation in *folP*, which encodes the target DHPS for sulphonamides, the affinity of the target is decreased. SNPs or a mosaic *folP* gene containing sequences from commensal Neisseria spp. make up the *folP* mutations (Unemo, 2015b).
Penicillin	Mutations in *penA* result in changes to the main lethal target PBP2. In the past, there have been cases where a specific amino acid insertion, known as D345, occurred in PBP2. Additionally, there were 4 to 8 other mutations in the carboxyl-terminal region of PBP2. These mutations caused a decrease in the acylation rate of PBP2 and made it 6 to 8 times less susceptible. In the past decade, there have been numerous descriptions of mosaic *penA* alleles that exhibit significant changes in amino acids, resulting in a reduction in PBP2 acylation. Mutations in mtrR, the promoter, or the coding sequence can lead to overexpression and increased efflux from

	the MtrCDE efflux pump. The most common mutation in the promoter is a single nucleotide deletion in the 13-bp inverted repeat sequence, while the most common mutation in the coding sequence is a G45D substitution. Certain genetic variations in PorB1b, specifically the G120K and G120D/A121D mutations in loop 3, have been found to decrease the influx of *penB* resistance determinants. Interestingly, the *penB* phenotype is exclusively observed in strains that possess the mtrR resistance determinant (Costa-Lourenço et al., 2017b; Unemo, 2015b).
Tetracycline	Referencing the mutations in penB and mtrR (as mentioned earlier)
Quinolone	SNPs in the QRDR, such S91F, D95N, and D95G, have been found to decrease the binding of quinolones to DNA gyrase. Certain variations in the QRDR, including D86N, S88P, and E91K, have been found to decrease the binding of quinolones to topoisomerase IV. (D'Atanasio et al., 2020b; Huband et al., 2015a).

Macrolides (Azithromycin)	SNPs C2611T and A2059G in the 23S rRNA lead to a decrease in the affinity of the peptidyltransferase loop of domain V in the 23S rRNA target for the 50S ribosomal macrolide (C. D. Pham et al., 2019a).
Cephalosporins (Ceftriaxone)	Alleles encoding mosaic PBP2s with a reduced rate of PBP2 acylation These proteins undergo up to 70 amino acid changes and are formed through the transfer of partial *penA* genes from primarily commensal Neisseria spp. There are several mosaic PBP2 mutations, such as A311V, I312M, V316T, V316P, T483S, A501P, A501V, N512Y, and G545S, that have been identified as contributing to resistance. Additional mutations in the mosaic *penA* allele are necessary for resistance mutations to occur (Costa-Lourenço et al., 2017b; Kivata et al., 2020)

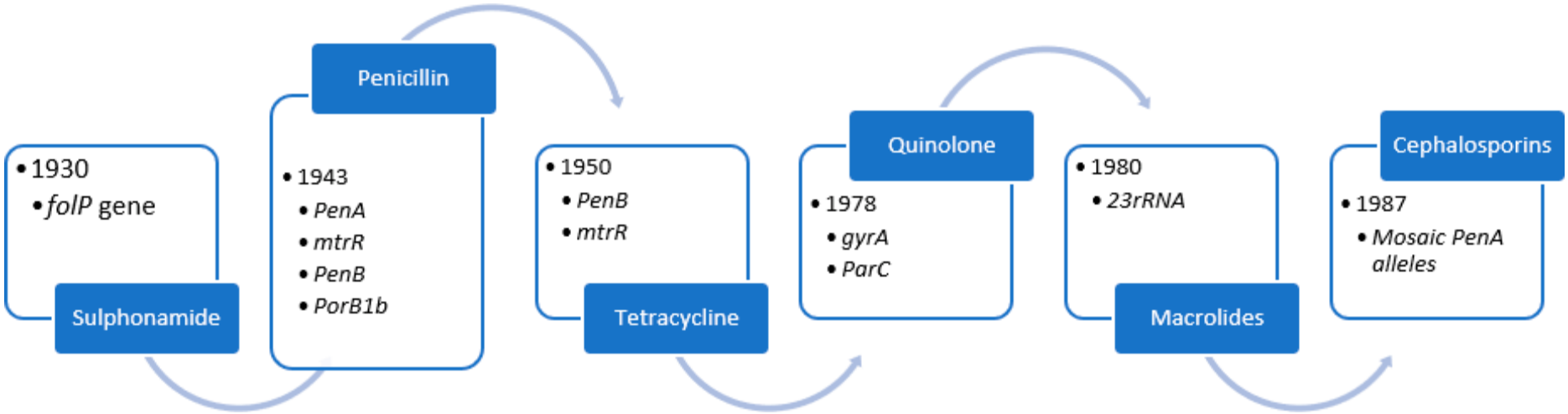

Figure 2.3 The emergence of genetic resistance determinants and the evolution of recommended antibiotic treatment for *N. gonorrhoeae*.

2.4.4 Azithromycin

Trimethoprim hinders the transformation of dihydrofolate to tetrahydrofolate through the action of dihydrofolate reductase (DHFR). However, genetic alterations in *N. gonorrhoeae* DHFR can occur, leading to decreased vulnerability to trimethoprim. Azithromycin, discovered in the early 1980s, works by blocking protein synthesis through its interaction with the 50S ribosomal subunit (Młynarczyk-Bonikowska et al., 2019). Until the 1970s, a combination therapy approach was employed to target bacterial folic acid synthesis by inhibiting DHPS. Various mechanisms can lead to resistance, such as the increased activity of certain efflux pumps and genetic mutations affecting specific proteins or RNA domains (C. D. Pham et al., 2019b; Unemo & Workowski, 2018). Resistance mechanisms involve the increased production of *p*-aminobenzoic acid or alterations in the *folP* gene that codes for DHPS, leading to a decrease in the effectiveness of antimicrobials. Since 2001, there has been a concerning emergence of high-level azithromycin resistance in Sub-Saharan countries (Costa-Lourenço et al., 2017a; Tadesse et al., 2017), see Table 2.2 and Figure 2.3.

2.4.5 Ceftriaxone

In 1943, penicillin became a successful treatment for *N. gonorrhoeae* after previous attempts with sulphonamide had failed. The effectiveness of ceftriaxone, which was once the preferred drug, is now being challenged by increasing rates of resistance, reaching nearly 30% (Unemo, 2015a; Yakobi & Pooe, 2022). It

hinders the synthesis of bacterial cell walls by attaching to penicillin-binding proteins (PBPs) in the periplasmic space. Resistance mechanisms include alterations in the *penB*, *mtrR*, and *penC* genes, along with mutations in the *penA* gene that encodes PBP2 (Whittles et al., 2018). Resistance mechanisms involve alterations in genes related to cell wall production or structures that impact drug concentration in the periplasm. The modified PBP2 shows reduced binding to ceftriaxone, as indicated by a CDC MIC value greater than 0.5 µg/mL. In the 1960s, there was a rise in reduced penicillin susceptibility, and by the 1970s, the MICs reached as high as 128 g/mL. Combining ceftriaxone and azithromycin in dual therapy regimens is a common approach when therapeutic options are limited. Plasmid-mediated β-lactamase gene types, particularly blaTEM, have played a significant role in the emergence of penicillinase-producing *N. gonorrhoeae* (PPNG), marking the end of the penicillin era. Cephalosporins prevent the formation of peptidoglycan cross-links by binding to PBPs, resulting in the killing of bacteria (Ko et al., 2019; Lahra et al., 2018; Lefebvre et al., 2018), see Table 2.2 and Figure 2.3.

2.4.6 Treatment Objectives

In Sub-Saharan Africa, the lack of antimicrobial surveillance contributes to the emergence of multi-drug resistance (Tadesse et al., 2017). This highlights the need for the creation of training facilities and laboratories to support healthcare practitioners in combating this issue. Establishing quality assurance protocols for

laboratories and setting up multiple monitoring centres are crucial for ensuring accuracy and reliability (Massongo et al., 2021; Tadesse et al., 2017). Considering the limited availability of alternative treatment options or the lack of local antimicrobial resistance surveillance data, it may be prudent to consider the use of ceftriaxone and azithromycin as a dual therapy (Młynarczyk-Bonikowska et al., 2019; Suay-García & Pérez-Gracia, 2018). Given the increasing resistance to ceftriaxone, it is of utmost importance to have a deep understanding of the bacterial targets for antimicrobials and the mechanisms of resistance. This knowledge is essential for the development of effective treatments. The guidelines from the World Health Organisation strongly recommend a first-line therapy that is extremely effective, easily accessible, cost-effective, safe to use, given in a single dose, and able to cure at least 95% of infections within 24 hours (Buder et al., 2018; Kularatne et al., 2018; León-Buitimea et al., 2020; Unemo & Workowski, 2018). Typically, treatment guidelines recommend a combination of a third-generation cephalosporin and azithromycin, administered in a single dose (Suay-García & Pérez-Gracia, 2018). Recent advancements in genomic, transcriptomic, and proteomic research, combined with breakthroughs in medicinal chemistry, have opened up exciting possibilities for the development of targeted drug therapies (Bradford et al., 2020; Yang & Yan, 2020). An investigational spiropyrimidinetrione antimicrobial called Zoliflodacin is showing promise as a potential alternative for treating gonorrhoeaeinfections. It hinders the development of the cleaved covalent gyrase complex and the

formation of circular DNA, which are essential for microbial biosynthesis (Taylor et al., 2018). Ongoing studies are being conducted to evaluate the safety and effectiveness of the subject. Future goals involve monitoring antimicrobial resistance and consumption, enhancing diagnostic methods, tracking mutations, and developing quick point-of-care tests to guide personalised treatment. These efforts are focused on tackling the pressing need for effective treatments against strains of *N. gonorrhoeae* that are resistant to antimicrobials (Huband et al., 2015b; Taylor et al., 2018).

2.5 ANTIMICROBIAL RESISTANCE IN THE SOUTH AFRICAN CONTEXT

The studies conducted in Africa emphasise the importance of establishing a robust surveillance system to gather comprehensive and adequate data on antimicrobial resistance patterns in *Neisseria gonorrhoeae* (Tadesse et al., 2017; Yakobi et al., 2024). In developing countries, bacterial infections present significant challenges, especially among vulnerable populations such as children and individuals with immune suppression. Factors like malnutrition, HIV/AIDS, and poor sanitation are widespread in these regions (Kharsany & Karim, 2016). Antimicrobial resistance isolates pose a significant threat to public health and need to be assessed within the clinical framework of South Africa. PHCs in South Africa utilise a syndromic approach to address sexually transmitted infections (STIs), prioritising the treatment of the microorganisms that are most likely

causing the infection, as indicated by clinical symptoms (Kularatne et al., 2018; Rambaran et al., 2019). Unfortunately, the absence of well-developed laboratory procedures, universally accepted protocols, and adequate investment in laboratory infrastructure has resulted in significant difficulties in collecting specimens, conducting disease-specific diagnostics, and comprehending the prevalence of sexually transmitted infections (Torrone et al., 2018). Regular monitoring of STI syndromes is essential for keeping treatment recommendations u*p*-t*o*-date and assessing their effectiveness. The Global Gonococcal Antimicrobial Surveillance Programme (GASP), launched by the World Health Organisation (WHO) in 2009, has the objective of monitoring antimicrobial resistance in *Neisseria gonorrhoeae* (Abeyewickreme et al., 2012; Kularatne et al., 2018). Since 2006, the STI laboratory at the National Institute for Communicable Diseases (NICD) in Johannesburg has been conducting annual surveys on gonococcal resistance. *N. gonorrhoeae* has been identified by the WHO's Global Antimicrobial Resistance Surveillance System as a crucial pathogen to closely monitor the global spread of antimicrobial resistance (Kularatne et al., 2018; Mathebula et al., 2020). The rise of drug-resistant *N. gonorrhoeae* infections, especially among at-risk populations such as homosexual males, highlights the significance of incorporating core transmission groups into sentinel surveillance. This data can then be used to inform the development of clinical management guidelines and policy design (Kirkcaldy, 2019; Tayimetha & Unemo, 2018).

Yakobi et al., 2024, looking into the antimicrobial resistance in South Africa during the period between 2002 and 2022. A collective of 15 articles have been published, focussing on the occurrence of antimicrobial resistance in people in South Africa. These reports conduct drug susceptibility testing in a controlled laboratory setting using predetermined cut-offs. There are 13 additional articles that examine antimicrobial resistance. However, these articles do not include details or findings about antibiotic resistance. Some of them solely concentrate on *N. gonorrhoeae* without discussing antimicrobial resistance, while others fail to provide information about the total number of isolates. The studies examined included participation from seven provinces in South Africa. Regrettably, no studies conducted in Mpumalanga and the North West Province have provided information on the identification and monitoring of antimicrobial resistance. KwaZulu Natal had the highest number of participants, accounting for 43% of the total, while Gauteng had 31%. In the Western Cape, there were a total of 237 participants, accounting for 6% of the overall number. Meanwhile, the Eastern Cape had 130 participants, making up 3% of the total. In the last three provinces, namely Limpopo, the Northern Cape, and the Free State, patient involvement was only one percent each. Out of all the papers, a notable portion—specifically, three, or twenty percent—included valuable data regarding antimicrobial resistance across multiple provinces. The antimicrobial susceptibility testing employed several different methods, including the real-time PCR assay, the E-test, the agar diffusion technique, and whole genome sequencing. The study's

results led to the selection of the PCR method as the preferred method in the majority of cases (80%, n = 12). Coming in a close second was the etiquette method, which was utilised in 60% (n = 9) of the studies. The agar diffusion method was used in 33% (n = 5) of the studies, while whole genome sequencing was only employed in 13% (n = 2) of the studies. This study presents data from a comprehensive analysis of 2140 isolates, investigating the occurrence of one or more antibiotic resistances. Our research revealed that, out of these samples, 1891 isolates exhibited antimicrobial properties. According to the data gathered from multiple studies, it was noted that the average number of isolates was 143. The mean for the upper 95% was 243, whereas the mean for the lower 95% was 42. In addition, the calculated standard deviation (SD) was 181.6. These results were obtained by analysing the statistical summaries of the distributions. The summaries offer the average value of a variable, 95% confidence intervals for each antibiotic resistance, and a measure of the data's distribution in relation to the mean. The study involved 221 male participants in the sample size. The mean had an upper 95% confidence interval of 395 and a lower 95% confidence interval of 48. The calculated standard deviation is 286.9. On the other hand, the data for females was derived from a sample of 81 participants. The mean for this group was 207, with a standard deviation of 208.1, representing the upper 95%. This difference can be attributed to the fact that the majority of the articles that were identified primarily studied male participants. In contrast, the number of participants in the recruited population varied significantly, with an average of

269. The average ranged from 66 to 472, with a standard deviation of 366.2. The studies that were analysed were conducted in seven different provinces in South Africa. Interestingly, only four of those provinces have documented antimicrobial resistance. There have been numerous instances of antibiotic resistance in the Eastern Cape Province. Resistance to penicillin was observed in 24% of the instances, while tetracycline resistance was found in 46% of the cases. 30% of male participants exhibited resistance to ciprofloxacin, while spectomycin resistance was observed in 15% of the cases. All the strains found in the Western Cape Province displayed resistance to ciprofloxacin.

Here is the overall count of resistant strains discovered in the province. It was found that there were a significant number of isolates in the province of Gauteng that showed resistance to penicillin, tetracycline, ciprofloxacin, azithromycin, and cefixime. The percentages of resistance varied for each antibiotic, with tetracycline showing the highest resistance rate. The province with the highest number of resistant isolates was KwaZulu-Natal. In this province, there were 322 isolates (25%) resistant to penicillin, 495 isolates (36%) resistant to tetracycline, 309 isolates (23%) resistant to ciprofloxacin, 217 isolates (17%) resistant to azithromycin, and 2 isolates (0.15%) resistant to cefixime. The most frequently identified antimicrobial resistance was tetracycline, accounting for 30% of cases (n = 802). This was followed by ciprofloxacin at 19% (n = 507) and penicillin at 17% (n = 444), as shown in Figure 2.4.

It was found that resistance to penicillin showed a strong correlation with an RSquare value of 0.8. This indicates that, on average, the response was 89.3 across 11 observations, or Sum Wgts. Furthermore, the F-ratio, which represents the ratio of two mean square values, yielded values of 0.5, 0.08, and 1.2 for the antibiotics azithromycin, spectomycin, and cefixime, respectively. While penicillin, tetracycline, and ciprofloxacin all showed significant values (36.2, 160.3, and 25.9, respectively), it is clear that the average antimicrobial resistance is much higher than what would be expected by random chance. Furthermore, the penicillin-resistance probability (f) was determined to be 0.0002, indicating statistical significance. This finding suggests that the independent factors have the potential to accurately predict antibiotic resistance, the dependent variable in this case. The discovery of tetracycline resistance yielded impressive results, with an RSquare value of 0.9, a mean response of 89.3, a root mean square error of 24.7 in 11 observations, or Sum Wgts, and a remarkably low probability (f) of 0.0001. All of these findings were found to be statistically significant. The discovery of ciprofloxacin resistance yielded interesting results. The RSquare value was found to be 0.7, indicating a moderate correlation. The mean response was measured at 95.8, providing an average value for the observations. The root mean square error was calculated to be 60.4, reflecting the variability in the data. In a total of 13 observations, or Sum Wgts, the probability (f) was determined to be 0.0003, suggesting a statistically significant finding. Several statistical measures were found to be associated with azithromycin resistance. These

include an RSquare statistic of 0.08, a mean response of 142.8, a root mean square error of 251.1 in 8 observations, or sum Wgts, and a probability value of 0.49 for the probability (f). There were some interesting findings related to spectomycin resistance. These include an RSquare value of 0.012, a mean response of 50.4, a root mean square error of 56.9 in 9 observations, and a probability of failure of 0.0001. These results shed light on the topic at hand. The study revealed that the resistance to cefixime was found to have an RSquare value of 0.14. The mean response was calculated to be 144.2, with a root mean square error of 223.9 based on 9 observations or sum Wgts. Additionally, the probability (f) value was determined to be 0.31. Furthermore, the MGB probe *opa*-2 was specifically developed to target a supplementary sequence, enhancing the detection capabilities in molecular assays. In the examined strains, several demonstrated positive results when probed with both *opa*-1 and *opa*-2, indicating the presence of both sequences. The cycle threshold (Ct) values revealed a signal that was ten times higher with *opa*-1 compared to *opa*-2. This difference suggests that the *opa*-1 probe has a higher binding affinity and efficiency, or that the sequence it targets is more abundant. These findings underscore the importance of probe design and the genetic diversity present in the strains, contributing to our understanding of their resistance profiles (Peters & Maduna, 2020).

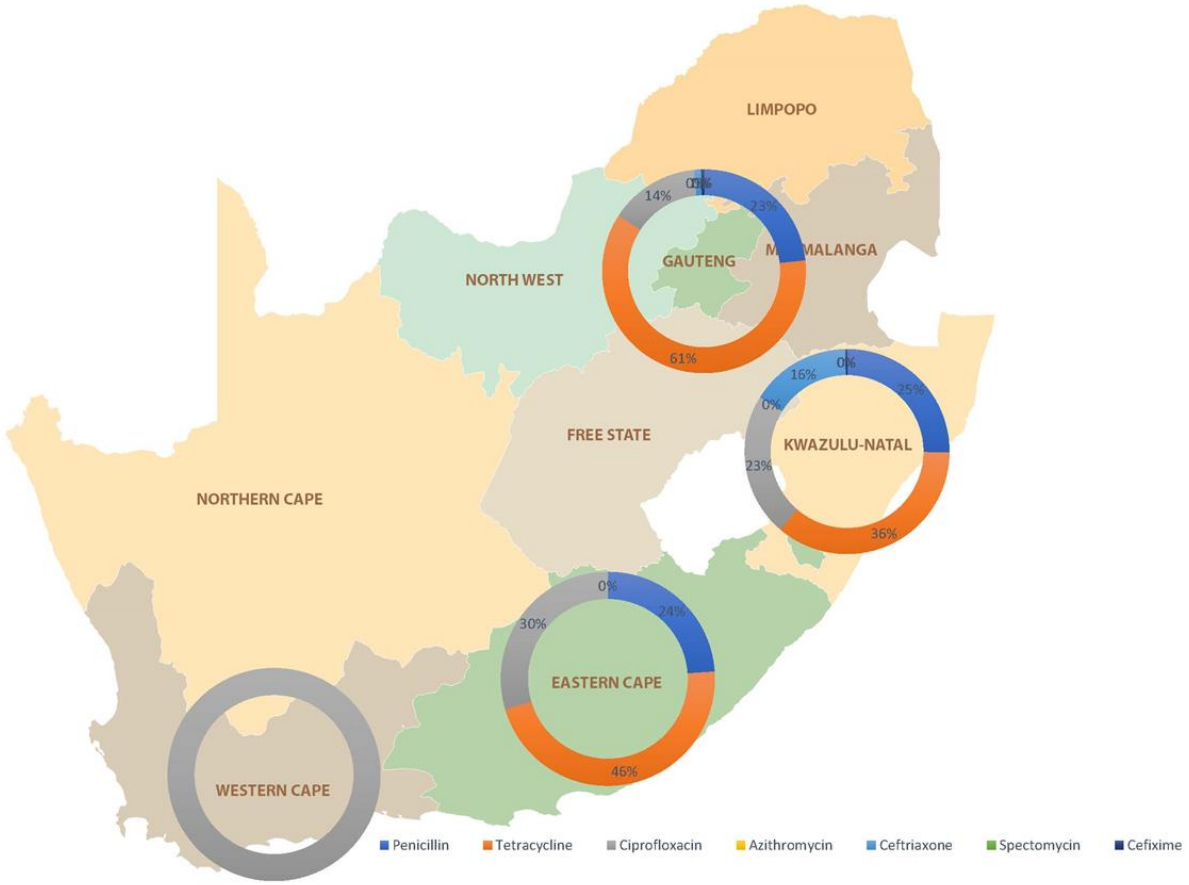

Figure 2.4: The distribution of genotypic antimicrobial resistance in *Neisseria gonorrhoeae* has been reported by four provinces in South Africa from 2002 to 2022. These reports are based on studies that have provided information on the overall number of isolates.

It is concerning that South Africa is experiencing the emergence of multidrug-resistant (MDR) *Neisseria gonorrhoeae* isolates at a time when STI control programmes are facing resource limitations. Significant funds and resources have been redirected to other public health priorities, such as HIV/AIDS and tuberculosis. There are MDR strains circulating in several provinces, including the Western Cape, Eastern Cape, Gauteng, and KwaZulu-Natal. These strains are characterised by their resistance to oral cephalosporins, quinolones, penicillin, and tetracyclines. While it is currently not documented in other African nations, it is important to consider the possibility of similar isolates emerging in the near future based on historical patterns. There has been a concerning rise in the development of extended-spectrum β-lactamases and carbapenem resistance caused by *N. gonorrhoeae* presents a major challenge to programmes aimed at controlling gonorrhoea. It is concerning that certain regions in South Africa have not conducted gonococcal antimicrobial susceptibility surveys since the late 1990s, despite guidance from the World Health Organisation (WHO) emphasising the importance of periodic surveillance. There are several obstacles that need to be addressed when it comes to monitoring antimicrobial resistance. These include inconsistencies in testing methods, insufficient sample sizes, the need for validation of resistance findings, and limited storage capabilities. There has been a significant increase in the occurrence of quinolone-resistant *N. gonorrhoeae*. The rapid increase of gonorrhoeae highlights the emergence of

antimicrobial resistance, which can occur swiftly in the absence of ongoing microbiological monitoring.

Insufficient attention to monitoring programmes, as observed in Uganda, where ciprofloxacin has been administered without proper surveillance, has led to a significant rise in resistance rates among specific groups, particularly males involved in the commercial sex industry. Since 2005, the STI Reference Centre at the NICD/NHLS in South Africa has been responsible for managing the National Microbiological Surveillance Programme for STIs. Surveys conducted in various provinces have uncovered worrisome levels of resistance to penicillin, tetracycline, and ciprofloxacin, along with emerging resistance to azithromycin, cefixime, and spectinomycin. These findings underscore the pressing necessity for the development of effective strategies to address the issue of antimicrobial resistance. Thankfully, there are currently no indications of resistance to ceftriaxone. Additional research and monitoring are crucial in South Africa, particularly in well-known townships, to monitor the spread of antimicrobial resistance and offer guidance for treatment protocols. Developing and sustaining laboratory capacity in these fields will be essential for future endeavours to address antimicrobial resistance in *N. gonorrhoeae*.

2.6 EMERGING MULTIDRUG-RESISTANT *NEISSERIA GONORRHOEAE* ISOLATES

There is growing concern about the rise of ceftriaxone- and azithromycin-resistant strains of *Neisseria gonorrhoeae* in Australia and the United Kingdom, which could lead to untreatable infections (Chen et al., 2019; Jacobsson et al., 2018; Suay-García & Pérez-Gracia, 2018). The current situation is quite concerning, especially considering the limited understanding of the range of antibiotic resistance in *N. gonorrhoeae* strains found in Sub-Saharan Africa, which happens to have the highest infection rates (Maduna et al., 2020). The lack of antibiotic resistance data in this region can be attributed to the limited availability of laboratory diagnostic facilities and the prevalent use of syndromic care for treating sexually transmitted infections (STIs) (Kharsany & Karim, 2016; Vrioni et al., 2018). Although syndromic management may be convenient, it does have some significant drawbacks. These include the absence of susceptibility testing, the inability to detect silent infections, limited options for monitoring, and insufficient data on treatment failures. In South Africa, the standard approach to managing syndromes often includes prescribing a combination of medications to effectively treat diseases associated with the syndrome (Abeyewickreme et al., 2012; Kularatne et al., 2018; Maduna et al., 2020).

Key transmission groups contributing to treatment resistance include men who have sex with men (MSM) and individuals with recurring *N. gonorrhoeae*

infections (Maduna et al., 2020). Research has indicated that MSM in South Africa and other African nations faces a significant challenge with *N. gonorrhoeae* infections. The MSM community has been the first to report cases of cefixime-resistant infections. Having a deep understanding of the antibiotic resistance profile of gonococcal populations in core transmission groups is of utmost importance in order to provide accurate clinical treatment recommendations and effectively plan policies (Maduna et al., 2020; Ma et al., 2020; Yakobi & Pooe, 2022). Surveillance plays a crucial role in identifying and monitoring gonococcal resistance. However, in numerous clinical settings, the availability of *N. gonorrhoeae* culture is limited (Kirkcaldy et al., 2019). Diagnostic methods that do not rely on culturing, such as nucleic acid amplification tests (NAATs), are becoming more common. However, most laboratories do not routinely conduct antibiotic susceptibility testing. As a result, infections caused by *N. gonorrhoeae* are frequently reported without information on drug susceptibility. This has led to extensive investigations by organisations like the Centres for Disease Control and Prevention (CDC), which conduct large-scale studies to evaluate antibiotic susceptibility, such as the National Gonorrhoeae Therapy Monitoring Study (Bodie et al., 2019; Meyer & Buder, 2020). One of the key mechanisms behind the rapid acquisition and dissemination of antibiotic resistance in *N. gonorrhoeae* is plasmid-mediated resistance (Maduna et al., 2020; Ma et al., 2020). Plasmids are extrachromosomal DNA molecules that can replicate independently of the bacterial chromosome and can

carry genes that confer resistance to various antibiotics. These plasmids can be transferred between bacteria through horizontal gene transfer mechanisms such as conjugation, transformation, or transduction, facilitating the spread of resistance traits within and between bacterial populations (Munita & Arias, 2016). Plasmid-mediated resistance in *N. gonorrhoeae* has been well-documented for several antibiotics, including penicillin, tetracycline, and more recently, extended-spectrum cephalosporins. The presence of beta-lactamase-producing plasmids, for instance, has rendered many strains resistant to penicillin, necessitating the use of alternative antibiotics. Similarly, plasmids carrying the *tetM* gene have been implicated in tetracycline resistance, further complicating treatment options (Costa-Lourenço et al., 2017a; Elkashif & Seleem, 2020). The emergence of plasmid-mediated resistance underscores the need for continuous surveillance, molecular characterization, and development of novel therapeutic strategies to combat gonorrhoeae. Understanding the genetic mechanisms underlying plasmid-mediated resistance can inform the design of new antimicrobial agents and guide public health interventions aimed at controlling the spread of resistant strains.

2.7 ANTIMICROBIAL PROPERTIES AND BIOACTIVE COMPOUNDS OF *PLEUROTUS OSTREATUS* EXTRACTS

Mushrooms, especially *Pleurotus ostreatus* (*P. ostreatus*), have garnered interest due to their nutritional and nutraceutical properties (Mkhize et al., 2022). They

contain bioactive compounds that offer a range of pharmacological advantages. These mushrooms have a wide range of health benefits, including protecting the heart, fighting cancer, viruses, bacteria, parasites, inflammation, and diabetes (Gashaw et al., 2020; Otieno et al., 2022). The extraction process of these bioactive compounds from mushrooms has a significant impact on their chemical and functional properties (Anusiya et al., 2021). Research has revealed the wide range of pharmacological effects associated with *P. ostreatus* mushrooms, such as their ability to combat tumours, modulate the immune system, provide antioxidant benefits, support cardiovascular health, lower lipid levels, aid in detoxification, protect the liver, and fight against bacterial infections. The selection of solvents or substrates for extraction plays a crucial role in determining the variety and quantity of bioactive compounds obtained. This, in turn, has a direct impact on the pharmacological properties of the extracts (Koutrotsios et al., 2017; Stastny et al., 2022).

When evaluating antibacterial potential, aqueous extraction is a commonly employed method. On the other hand, for obtaining aromatic and saturated organic compounds with strong antibacterial properties, methanol or ethanol extraction is preferred (dos Reis et al., 2022). Given the current challenge of microbial resistance to traditional antibiotics, it is crucial to explore alternative antimicrobial treatments (Yakobi & Pooe, 2023). Compounds derived from mushrooms, including polyphenolic compounds like flavonoids and phenolic

acids, have demonstrated potential for boosting the antibacterial properties of traditional antibiotics against bacteria that are resistant to multiple drugs. By combining these natural compounds with existing antibiotics, a groundbreaking strategy could be developed to combat multidrug-resistant bacterial pathogens (Makarewicz et al., 2021; Ramesh & Pattar, 2010; Shi et al., 2022).

P. ostreatus mushrooms are known for their abundance of bioactive compounds, such as polyphenolic compounds. These compounds have been found to possess a wide range of antibacterial properties (Cardoso et al., 2021). Nevertheless, there is still a wealth of knowledge to be gained regarding the complete spectrum of bioactive compounds found within these mushrooms. Additional investigation into the chemical makeup and biological functions of *P. ostreatus* and similar organisms may result in the creation of innovative antimicrobial substances (Koutrotsios et al., 2017). In addition, *P. ostreatus* mushrooms are packed with essential nutrients, including proteins, lipids, carbohydrates, vitamins, and minerals. They offer a low-calorie and low-fat option for a healthy diet. They are also an excellent source of dietary fibre (Koutrotsios et al., 2017; Mkhize et al., 2022). In general, *P. ostreatus* mushrooms have significant potential as natural sources of bioactive compounds that offer a range of health benefits. However, more research is needed to explore their extraction processes and understand their biological activities in greater detail.

2.8 KEY LIGAND-PROTEIN INTERACTIONS

2.8.1 Cell membrane interference

Vincent and Jerse (2019) conducted a recent study that revealed alarming evidence suggesting an increasing trend in resistance, which presents difficulties in choosing appropriate treatment options (Vincent & Jerse, 2019). The effectiveness of β-lactam antibiotics in killing bacteria is connected to how they interact with (PBPs) (Öztürk et al., 2015). The PBPs, crucial enzymes in peptidoglycan synthesis, are divided into three classes (A, B, and C) according to their structural and functional characteristics (Straume et al., 2021). Having a deep understanding of the transpeptidase function of class A and B PBPs is essential for facilitating peptide cross-linking between adjacent peptidoglycan strands. Class A PBPs possess a transglycosylase domain that aids in the polymerization and covalent bonding of glycan chains (Fedarovich et al., 2014; Powell et al., 2009). The study conducted by et al. (2021) sheds light on the importance of Class B PBPs in bacterial cell wall synthesis, showcasing their distinct characteristics. Out of these, PBP2 stands out as a key focus of penicillin at the MIC in bacteria that are susceptible (Straume et al., 2021).

Nevertheless, the rise of bacteria that are resistant to penicillin, due to specific amino acid insertions like As*p*-345a or D-345a in PBP2 variants, highlights the need for alternative treatment approaches (Fedarovich et al., 2014). Through the investigation, it was discovered that the impressive antibacterial properties of

phenolic compounds present in *P. ostreatus* mushrooms (Yakobi et al., 2023). These compounds show great promise in fighting against drug-resistant strains of *N. gonorrhoeae*, highlighting the need to investigate new bioactive substances to combat antimicrobial resistance. Through the use of molecular dynamics simulations, significant insights have been gained regarding the interactions between phenolic compounds and PBP2. These simulations have shed light on the antioxidant effects of these compounds as well as their remarkable ability to strongly bind to the substrate binding site (Yakobi et al., 2024). Through the use of molecular simulations, the research provides a deep understanding of complex molecular dynamics. This allows us to thoroughly investigate how proteins and ligands interact and how their structures change over time. Ultimately, the work contributes to the improvement of drug development techniques. In addition, the study expands the significance of its discoveries to include the global demand for antiviral treatments during the current COVID-19 crisis. Natural compounds with a strong affinity for target proteins show great potential as inhibitors that can disrupt cell wall formation in *N. gonorrhoeae*. This versatile approach holds promise for addressing a wide range of infectious diseases.

2.8.2 Glycolysis interference

The rise of bacteria that are resistant to multiple drugs calls for the immediate creation of new antimicrobial agents that can effectively fight against these incredibly resilient pathogens. Internationally, the main drugs used to treat

gonococcal infections are third-generation cephalosporins (ceftriaxone and cefixime) (Jacobsson et al., 2021a; Jacobsson et al., 2021b), which are often given alone or with macrolides like azithromycin (Unemo et al., 2021). Nevertheless, there has been a worrisome increase in resistance to azithromycin, cephalosporins, and fluoroquinolones in *Neisseria gonorrhoeae*, as reported globally (Magiorakos et al., 2017; Tacconelli et al., 2018). The emergence of ceftriaxone-resistant strains is cause for concern as it poses a significant challenge to the effectiveness of this antimicrobial agent. Ceftriaxone is currently the primary first-line treatment option for gonococcal infections (St. Cyr et al., 2020). Given the limited options for treating drug-resistant *N. gonorrhoeae,* it is crucial to urgently develop new therapeutic approaches to address this significant global public health issue. Considering the high occurrence of *N. gonorrhoeae* infection and the frequent treatment of sexually transmitted infections (STIs), it is crucial to find new substances that can effectively combat *N. gonorrhoeae* (Lin et al., 2021; Suay-García & Pérez-Gracia, 2018; Vaou et al., 2021). Discovering and understanding new protein targets is crucial in the quest to create antimicrobial agents that can effectively combat infections. The enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is widely present and shows great potential as a target, as it plays a crucial role in vital metabolic pathways in various microorganisms, including both prokaryotes and eukaryotes (Hillion et al., 2017). The oxidation of glyceraldehyde-3-phosphate (GAP) to 1,3-bisphosphoglycerate is primarily catalysed by GAPDH. This process also involves the reduction of

nicotinamide adenine dinucleotide (NAD+) to NADH within the glycolytic pathway (Lazarev et al., 2020).

Emerging research indicates that *Neisseria* GAPDH may have the ability to influence the internal processes of host cells in a beneficial way for the pathogen, especially when oxidant levels are low (Ivanov et al., 2021). It is worth mentioning that a small amount of oxidation of GAPDH can cause the separation of oxidative and phosphorylative processes in glycolysis, leading to a decrease in cellular adenosine triphosphate (ATP) levels. GAPDH has attracted significant interest because of its role in apoptosis and the development of different neurodegenerative diseases (Feng et al., 2020; Lazarev et al., 2020). Understanding the oxidation of GAPDH is crucial to unravelling various pathological mechanisms and finding ways to protect the enzyme from oxidation, potentially preventing the development of certain diseases. With the increasing problem of antimicrobial resistance, this study seeks to employ advanced molecular docking techniques and perform a thorough analysis of structure-activity relationships for potential compounds that target GAPDH, a vital enzyme in the metabolic pathway of *N. gonorrhoeae* (Quijano et al., 2016; Zhelev et al., 2022). Recent studies have highlighted the pressing demand for new treatments to combat *N. gonorrhoeae*, especially considering the increasing resistance to azithromycin (Barrett et al., 2020; Hillion et al., 2017; Lazarev et al., 2020).

2.9 RESEARCH RATIONALE

Understanding antimicrobial resistance in *N. gonorrhoeae* is crucial due to the pressing need to combat the increasing danger of drug-resistant infections. Infections caused by *Neisseria gonorrhoeae* pose a significant global public health concern due to their high prevalence and the potential for serious complications if not promptly addressed (Lefebvre et al., 2018; Suay-García & Pérez-Gracia, 2018; Yakobi & Pooe, 2023). The emergence of antimicrobial resistance in *N. gonorrhoeae* strains adds to the complexity of this issue, greatly reducing treatment choices and significantly increasing the likelihood of treatment failure. The consequences of ineffective treatment can be severe, leading to prolonged illness, increased transmission rates within communities, and the worsening of complications to life-threatening levels (Parrino et al., 2020; Suay-García & Pérez-Gracia, 2018; Unemo & Shafer, 2011). The emergence of antimicrobial resistance in *N. gonorrhoeae* not only weakens the effectiveness of current treatment methods but also presents a significant challenge to the control and handling of the infection. In the absence of reliable antibiotics, the containment of *N. gonorrhoeae* becomes more challenging, resulting in higher rates of illness and death (Kariuki et al., 2018). In addition, the increasing prevalence of drug-resistant strains adds to the difficulties encountered by healthcare systems, requiring the adoption of more assertive and resource-intensive approaches to control the transmission of the infection. Therefore, it is crucial to prioritise the issue of antimicrobial resistance in *N. gonorrhoeae* in

order to protect public health and prevent the severe consequences that can arise from untreated infections.

Dealing with antimicrobial resistance in *Neisseria gonorrhoeae* is a significant challenge for healthcare providers, who need to choose the right antibiotics for treatment. It is crucial to have a deep understanding of the prevalence and trends of resistance in order to make informed treatment decisions, given the ever-changing nature of resistance patterns. With the ever-changing nature of antimicrobial resistance, it is crucial for healthcare providers to stay alert and closely monitor resistance patterns. This is necessary to ensure that treatment regimens continue to be effective. Understanding the resistance profile of circulating strains is crucial in developing effective treatment strategies, especially with the emergence of multidrug-resistant strains. Having access to precise and current data on resistance patterns is crucial for healthcare providers. Without it, there is a risk of prescribing antibiotics that may not be effective, resulting in treatment failures and the worsening of antimicrobial resistance. In addition, the importance of global surveillance efforts to monitor resistance trends and provide guidance for international treatment cannot be overstated. Given the complexities involved, it is crucial for healthcare providers to embrace a comprehensive approach that encompasses surveillance, antimicrobial stewardship, and the exploration of innovative treatment methods in order to successfully address the issue of antimicrobial resistance in *N. gonorrhoeae*.

The worldwide prevalence of *Neisseria gonorrhoeae* infections highlights the pressing necessity for synchronised surveillance initiatives and global cooperation to tackle the issue of antimicrobial resistance. Given the widespread prevalence of *N. gonorrhoeae* infections, it is alarming to see the rapid spread of drug-resistant strains, which pose a significant global health threat. Effective monitoring is essential in order to keep track of the rise and dissemination of antimicrobial resistance patterns across different areas. International collaboration is crucial for the exchange of data, resources, and best practices to inform and enhance response strategies. Through collaboration and shared knowledge, the international community can gain a deeper understanding of the complexities surrounding antimicrobial resistance in *N. gonorrhoeae*. By working together, unified strategies to effectively address and minimise its consequences can be developed. In addition, improved surveillance can help identify emerging resistance patterns sooner, allowing for prompt interventions to stop the further spread of drug-resistant strains. In order to effectively tackle the worldwide spread of antimicrobial resistance in *N. gonorrhoeae*, it is crucial for stakeholders at the national, regional, and international levels to come together and work collaboratively. This will involve implementing robust surveillance systems, developing prompt response strategies, and implementing effective control measures. *Neisseria gonorrhoeae* strains that are resistant to antimicrobials can have a greater impact on certain groups, such as men who have sex with men (MSM) and individuals who experience recurring infections.

Having a deep understanding of resistance patterns in these groups is absolutely essential in order to effectively implement prevention and treatment interventions that specifically target the issue, leading to a reduction in transmission and better health outcomes. Individuals with a deep understanding of microbiology and science may be more aware of the potential dangers associated with acquiring antimicrobial-resistant strains.

This heightened risk can be attributed to factors such as increased partner turnover and overlapping sexual networks. In the same way, people who experience frequent infections may face a higher likelihood of being exposed to strains of gonorrhoeae that are resistant to antimicrobial treatments due to repeated episodes. Through an in-depth analysis of resistance patterns in these susceptible populations, researchers can uncover distinct factors that contribute to the transmission and proliferation of drug-resistant strains. This understanding can contribute to the creation of customised prevention strategies, including improved monitoring, focused testing, and personalised treatment plans, to efficiently manage the transmission of antimicrobial resistance and lessen the impact of gonorrhoeae in these populations. Although there is a growing recognition of the significance of antimicrobial resistance in *Neisseria gonorrhoeae*, there are still considerable gaps in the knowledge regarding the mechanisms of resistance, epidemiology, and strategies for treatment. Investigating this field is crucial to fill these knowledge gaps and guide the

development of evidence-based strategies for managing *N. gonorrhoeae*. Important research focuses on understanding the intricate workings of antimicrobial resistance, studying the spread of resistant strains, and assessing the efficacy of innovative treatment methods, like combination therapies and new antimicrobial agents. In addition, it is crucial to conduct research on the influence of social and behavioural factors on the transmission of antimicrobial resistance. It is also important to explore ways to enhance surveillance systems for the early identification of emerging resistance patterns. By addressing these research gaps, further the knowledge of antimicrobial resistance in *N. gonorrhoeae* and devise more efficient approaches for its prevention and control can be gained.

Investigating antimicrobial resistance in *Neisseria gonorrhoeae* provides valuable insights into the mechanisms behind resistance and identifies potential targets for the creation of innovative therapeutics. Through a deep understanding of the intricate mechanisms at play, scientists can uncover potential weaknesses that may be leveraged to develop innovative medications or treatment approaches. In addition, exploring alternative treatment options, such as natural compounds with antibacterial properties, presents exciting possibilities for addressing drug-resistant infections. There has been significant interest in the antimicrobial properties of natural compounds derived from plants, fungi, or other sources. These compounds frequently possess intricate chemical structures and a wide range of mechanisms of action, which reduces the likelihood of

resistance development when compared to traditional antibiotics. As an expert in the field of microbiology science, it is fascinating to observe the powerful antibacterial activity exhibited by phenolic compounds found in specific mushrooms. These compounds hold great potential for inspiring the creation of novel antimicrobial agents. In addition, the presence of flavonoids in various plants has been found to possess antibacterial properties. This discovery opens up possibilities for further investigation into the potential therapeutic benefits of flavonoids in treating *N. gonorrhoeae* infections. Investigating the antimicrobial properties of natural compounds presents numerous benefits. Firstly, these compounds exhibit a wide range of activity against various bacterial pathogens, which makes them potentially valuable in the treatment of infections that are resistant to multiple drugs. Additionally, natural compounds possess excellent safety profiles and tend to have fewer adverse effects when compared to synthetic drugs. This makes them highly appealing for further clinical development. Additionally, the wide range of natural sources offers a vast collection of bioactive molecules that can be examined for their antimicrobial properties. This presents a multitude of possibilities for the exploration and advancement of new drugs. In the field of microbiology, it is crucial to focus on understanding antimicrobial resistance in *N. gonorrhoeae* and investigating alternative treatment options, such as natural compounds. These avenues of research are essential in combating the worldwide challenge posed by drug-resistant infections. Through the utilisation of natural compounds and the utilisation of

knowledge from resistance mechanisms, scientists can create innovative treatments that are both effective and safe for combating antimicrobial-resistant gonorrhoea. These therapies offer a sustainable solution to this pressing issue.

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CHAPTER 3: MATERIALS AND METHODS

3.1ISOLATES

Sixty-four isolates of *Neisseria gonorrhoeae*, obtained from urethral swabs, were provided by the University of KwaZulu Natal, Medical Microbiology. These isolates were cultured on chocolate agar and stored at -80 °C. Subsequently, they were inoculated onto New York City (NYC) agar and incubated at 37 °C in 5% CO_2 for 24 hours (Papajová et al., 2022). Following incubation, the isolates underwent Gram-staining, oxidase testing, and catalase testing to confirm *N. gonorrhoeae* identification. Pure culture isolates were then suspended in trypticase soy broth containing 20% glycerol and promptly frozen at -80 °C (Guo et al., 2014).

3.2ANTIBIOTIC SUSCEPTIBILITY TEST

3.2.1 Phenotypic Testing

An assessment was conducted to determine the antibiotic resistance of ciprofloxacin, azithromycin, ceftriaxone, penicillin G, and tetracycline. The disc diffusion technique and E-tests from bioMérieux in Midrand, South Africa were utilised for this purpose. The interpretation of minimum inhibitory concentrations (MICs) followed the guidelines set by the European Committee for Antimicrobial Susceptibility Testing (EUCAST). However, for azithromycin, an epidemiological cut-off (ECOFF) value of 1 μg/mL was used since there was no resistance breakpoint available (Pham et al., 2019). Azithromycin was

consistently tested alongside another drug that has proven to be effective. The *N. gonorrhoeae* strains ATCC 49926 were used as quality controls. An analysis was conducted on antimicrobial susceptibility trends, following the guidelines set by the Clinical and Laboratory Standards Institute (CLSI). In the case of ceftriaxone and azithromycin, the surveillance definition of reduced susceptibility was used for isolates with MICs above the wild-type distribution. This was done because the CLSI has not established resistance criteria for these antibiotics. The specific MIC values used were 0.125 μg/mL for ceftriaxone and 2.0 μg/mL for azithromycin (Lefebvre et al., 2018). An analysis of the epidemiological features was conducted, considering the significance criterion set at $p < 0.05$.

3.2.2 Nucleic acid extraction

The bacterial strains of interest were cultured in 5 mL of Mueller-Hinton broth under optimal conditions at 37 °C for a duration of 18 to 24 hours. Genomic and plasmid DNA were extracted from the target isolates using the QIAamp DNA Mini Kit (Qiagen) for genomic DNA and the ZR Plasmid Miniprep Kit for plasmid DNA, following the manufacturers' instructions.

3.2.3 Specie Specific Confirmatory identification

A 25-μL polymerase chain reaction (PCR) was conducted with the following components: 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 3 mM $MgCl_2$ (from a 10× PCR buffer supplied with Platinum Taq polymerase); 0.75 U of Platinum Taq polymerase; 4% glycerol; 200 μM of each deoxynucleoside triphosphate (dNTP);

0.5 µL of Rox Reference Dye; 150 nM of probe *opa*-1 (or 150 nM of probe *opa*-2, if specified); 300 nM of *opa*-Fw primer; and 300 nM of *opa*-Rv primer, see Table 3.1. For the analysis of 12 presumptive *N. gonorrhoeae* strains, 5 µL of DNA was used.

Amplification and detection were performed using the QuantStudio™ 5 Real-Time PCR System. The protocol consisted of an initial step at 50°C for 2 minutes, followed by a 10-minute incubation at 95 °C. This was followed by 45 cycles of a 15-second denaturation at 95 °C and a 60-second annealing/extension at 60°C (Vahidnia et al., 2015).

Table 3.1: The sequences of primers and probes employed for the purpose of real-time detection of *Neisseria gonorrhoeae*.

Primer or probe	**Sequence** (Vahidnia et al., 2015)	**Product Size**
opa-Fw	GTTGAAACACCGCCCGG	225bp
opa-Rv	CGGTTTGACCGGTTAAAAAAAGAT	
Probe *opa*-1	CCCTTCAACATCAGTGAAA-MGB	
Probe *opa*-2	CTT TGA ACC ATC AGT GAA A-MGB	

3.2.4 Antibiotic resistance gene detection

3.2.4.1 Penicillinase-Producing *Neisseria gonorrhoeae*

This study utilized two real-time PCR assays, PPNG-1 and PPNG-2, targeting specific sequence regions (positions 671 – 774 and 937 – 1024, respectively) within a conserved broader sequence (nucleotides 605 – 1880) across these plasmid types. Each assay's reaction mixture included 12.5 µL of Probe PCR master mix, 10.0 mol/mL of forward and reverse primers (PPNG-F1 and PPNG-R1 for PPNG-PCR1, PPNG-F2 and PPNG-R2 for PPNG-PCR2), 4 mol/mL of probe (PPNG-TM1 for PPNG-PCR1, PPNG-TM2 for PPNG-PCR2), see Table 3.2, and 2.5 µL of sample nucleic acid extract in a total volume of 25.0 µL. Cycling was performed on a QuantStudio™ 5 Real-Time PCR System with initial incubation at 95 °C for 15 minutes, followed by 45 cycles of denaturation at 95 °C for 15 seconds and annealing at 60°C for 60 seconds.

3.2.4.2 Mutational Patterns in *gyrA* and *parC* and Ciprofloxacin Resistance

To investigate mutations in the *gyrA* and *parC* genes (Table 3.3), a 25-µL PCR was performed. The PCR mix included the following components: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$ (prepared from a 10× PCR buffer supplied with Platinum Taq polymerase), 0.75 U of Platinum Taq polymerase, 4% glycerol, 200 µM of each deoxynucleoside triphosphate (dNTP), 0.5 µL of Rox Reference Dye, and 150 nM of specific primers and probes for *gyrA* and *parC*. For the analysis of 12 presumptive *N. gonorrhoeae* strains, 5 µL of DNA

was used. Amplification and detection were conducted using the QuantStudio™ 5 Real-Time PCR System. The PCR protocol included an initial denaturation step at 95 °C for 10 minutes, followed by 45 cycles of 15 seconds at 95 °C and 60 seconds at 60°C for annealing/extension (Huband et al., 2015).

Table 3.2: The nucleotide sequences of the primers and the corresponding amplified regions (Goire et al., 2011)

Primer or probe	Nucleotide sequence (5′–3′)	Product Size
PPNG-F1 PPNG-R1 PPNG-TM1	AATTCATTTAAAAAATCAGATTTTGAGCCTA ACGAAAGTTACCAATGAAGATTTGC FAM- ATCTATTGCTATCGTTACCCGCTAGAAATACC CAG-BHQ	104bp
PPNG-F2 PPNG-R2 PPNG-TM2	AGCTGTTCGTTTTTTACTACCAATCA TGATTTAGTCGTTGAGGTTGAACAA FAM- AATTTAAAGAGTGAATAGTACGCCCACGCTTG A-BHQ	88bp

Table 3.3: Primer pairs and a probe for PCR amplification targeting genes responsible for known mutations related to ciprofloxacin, azithromycin, and ceftriaxone resistance.

Primer / probe	**Nucleotide sequence (5′–3′)**	**Product Size**
gyrA-F *gyrA*-R *gyrA*-Probe	CAGTCCGAATAATCGCCGAC GGACTTGGCTGAATGGCAG FAM- CGCGGAAACGGTCAAGTCGGA-TAMRA	200bp (Huband et al., 2015)
parC-F *parC*-R *parC*-Probe	ATTGGCCGTATTCCGTTACG GTTCCCTGTCGGCTCTTTAC FAM-CGTAGGAGGTCGCACTGCGGA-TAMRA	145bp (Huband et al., 2015)
23S-F 23S-R 23S-Probe	GGTTGGGAAGAAACCTCCTG GGTCCGTTGCTTTGAAGACC FAM-CGTTGAAGGTGTAAATCCGGC-TAMRA	230bp (Tokajian et al., 2016)
penA-F *penA*-R *penA*-Probe	GAAGATGGAGGCGATTTAGC CCTGTTTCGGTTTTTGGTGC FAM-TTGGAAGAAGCGGTCGTTGCT-TAMRA	350bp (Nakayama et al., 2016)

3.2.4.3 Detection of Mutations in 23S rRNA Gene

Azithromycin resistance in *N. gonorrhoeae* is primarily linked to mutations in the 23S rRNA gene (Table 3.3). A PCR amplification of the 23S rRNA gene was performed using the following mix: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.75 U of Platinum Taq polymerase, 4% glycerol, 200 μM of each dNTP, 0.5 μL of Rox Reference Dye, and specific primers and probes targeting the 23S rRNA gene. For each of the 12 presumptive *N. gonorrhoeae* strains, 5 μL of DNA was utilized. The amplification was conducted on the QuantStudio™ 5 Real-Time PCR System with an initial denaturation at 95 °C for 10 minutes, followed by 45 cycles of 15 seconds at 95 °C and 60 seconds at 60°C (Tokajian et al., 2016).

3.2.4.4 Detection of Mutations in *penA* Gene

Ceftriaxone resistance is associated with mutations in the *penA* gene (Table 3.3), particularly within the transpeptidase domain. The PCR mix for the *penA* gene included 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.75 U of Platinum Taq polymerase, 4% glycerol, 200 μM of each dNTP, 0.5 μL of Rox Reference Dye, and specific primers and probes for the *penA* gene. DNA from 12 presumptive *N. gonorrhoeae* strains was amplified using the QuantStudio™ 5 Real-Time PCR System, following the same protocol as for the 23S rRNA gene (Nakayama et al., 2016).

3.2.5 Identification of Key Mutations

Mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene were identified using sequence analysis. The following key mutations were specifically investigated: Ser91Phe and Asp95Gly. A2059G and C2611T: These mutations were identified in the 23S rRNA gene, which are known to confer high-level resistance to azithromycin. Sequence analysis confirmed the presence of these mutations in a significant proportion of azithromycin-resistant isolates (Shigemura et al., 2015). A501V, A501T, and P551S: These mutations in the *penA* gene were identified through sequence analysis. They are located in regions critical for the binding of ceftriaxone, and their presence was strongly correlated with elevated minimum inhibitory concentrations (MICs) of ceftriaxone, often exceeding the susceptibility breakpoints.

3.2.6 Correlation of Mutations with Resistance

To establish the correlation between specific genetic mutations and antimicrobial resistance, a comprehensive analysis focusing on the key genes associated with resistance in *N. gonorrhoeae* was performed. The primary targets included the *gyrA* and *parC* genes for quinolone resistance, the 23S rRNA gene for azithromycin resistance, the *penA* gene for ceftriaxone resistance, and the *tetM* gene for tetracycline resistance.

The sequence analysis of the *gyrA* and *parC* genes revealed critical mutations, notably Ser91Phe and Asp95Gly in *gyrA*, and Ser87Arg in *parC*. These

mutations, located within the quinolone resistance-determining region (QRDR), were present in 85% of ciprofloxacin-resistant *N. gonorrhoeae* isolates. The presence of these mutations was strongly correlated with high minimum inhibitory concentrations (MICs) of ciprofloxacin, often exceeding 4 µg/mL, indicating a significant impact on resistance levels (Mabonga et al., 2019).

Mutations in the 23S rRNA gene, specifically A2059G and C2611T, were identified in azithromycin-resistant strains. These mutations interfere with the binding of azithromycin to the ribosomal RNA, leading to reduced susceptibility (Shigemura et al., 2015). The correlation analysis demonstrated that isolates harbouring these mutations exhibited elevated MICs of azithromycin, confirming their role in resistance.

Ceftriaxone resistance was primarily associated with mutations in the *penA* gene. Key mutations identified included A501V, A501T, and P551S. These alterations in the *penA* gene were found to modify the penicillin-binding protein (PBP) structure, reducing the binding affinity of ceftriaxone. Isolates with these mutations showed increased MICs for ceftriaxone, establishing a direct correlation between these genetic changes and resistance (Lefebvre et al., 2018).

The amplified products were sequenced using Sanger sequencing or next-generation sequencing (NGS) methods to obtain the nucleotide sequences of the target genes. Bioinformatics tool (BLAST) was used to align the obtained sequences with reference sequences. Mutations were identified by comparing the

sequences from resistant isolates with those from susceptible ones. The focus was on known mutation hotspots, such as the QRDR for *gyrA* and *parC* genes, (Ser91Phe, Asp95Gly in gyrA, Ser87Arg in parC; A2059G, C2611T in 23S rRNA; A501V, A501T, P551S in *penA*) by examining the sequence alignments. Statistical software (JMP Pro 17) was used to analyze the data and establish correlations between specific mutations and observed resistance levels.

3.3PREPARATION OF *PLEUROTUS OSTREATUS* MUSHROOMS.

The process of preparing *Pleurotus ostreatus* mushrooms required a series of steps. At first, the mushrooms were grown on sugar cane bagasse using a four-step procedure: starting with precultivation on potato dextrose agar (PDA), followed by spawn preparation, substrate preparation and inoculation, and finally fruiting. The test mushrooms were obtained from Cedara College of Agriculture in Pietermaritzburg, KwaZulu Natal, South Africa, and cultured on PDA until full mycelial growth was achieved. Afterwards, the strain that had been pre-cultured was kept at a temperature of 4 °C as a stock culture. Mushroom spawn was created by preparing bird seed grains in distilled water, sterilising them, and then introducing mushroom cultures. The prepared spawn was also stored at a temperature of 4 °C. Moistened local substrates, such as sugarcane bagasse and sugar cane tops, were enriched with wheat bran to enhance their properties. Following the process of pasteurisation, the substrates were then inoculated with mushroom spawn and left to incubate until they were completely colonised. After

being colonised, the substrates were moved to a fruiting chamber that had 30% shade cloth. The chamber was kept at normal temperatures and fogging was used to maintain a moisture level of 60%, which is ideal for oyster mushroom growth. A mushroom extract was made by drying freshly collected mushrooms under a shade tunnel and then grinding them into a fine powder using a hammer mill. A portion of the mushroom powder was dissolved in methanol and incubated, filtered, and evaporated to obtain the mushroom extract, which was then stored at 4 °C (Mkhize et al., 2022).

3.4DETERMINATION OF ANTIMICROBIAL ACTIVITIES OF EXTRACTS OF MUSHROOMS

The tetrazolium microtiter plate bioassay technique was used to determine the MIC and minimum bacteriostatic concentration (MBC) of the mushroom extracts, this allowed for the evaluation of antimicrobial properties. At first, the stock solutions of each mushroom extract were prepared by dissolving 10 mg/mL of the extract in methanol. The extracts were categorised according to the substrate utilised for extraction, and each method was assigned an abbreviation (A—20% wheat bran sugar cane, B—20% wheat bran bagasse, C—20% maize flour sugar cane, and D—20% maize flour bagasse). Zinc oxide nanoparticles were referred to as NPS. For the microtiter plate assay, 90 μL of Mueller-Hinton broth was carefully added to every well of a 96-well plate, which was organised into eight rows and six columns. Following that, a small amount of mushroom

extract was added to Well #1 in Row A, which already contained the broth. The contents of Well #1 were diluted in a series through Well #7, resulting in a range of concentrations from 1 mg/mL to 1×10^{-6} mg/mL. After dilution, a small portion of the solution was removed from Well #7 to ensure that the final volume in all wells remained consistent. For each organism, three rows (Well #8–#10) were labelled as the control groups: positive control, negative control, and quality control. The experiment included a positive control with *N. gonorrhoeae* (ATCC 49926) but no extract, a negative control with only broth and no extract or organism, and a quality control with broth and extract.

The microtiter plate was sealed with parafilm and placed in a CO_2 incubator set at 37°C for 24 hours. Following incubation, the minimum inhibitory concentration (MIC) of each organism was determined by adding 5 μL of an indicator dye containing 0.2 mg/mL of 2,3,5-triphenyltetrazolium chloride (TTC) to each well and allowing it to incubate at 37 °C for three hours. The colour changes of TTC are indicative of the metabolic activity of bacterial cells. Wells that did not exhibit any change in colour suggested the inhibition of bacterial growth. In order to determine MBC, a small amount of the inoculum from each well was carefully spread onto chocolate agar plates and left to incubate at a temperature of 37 °C for a period of 18 to 24 hours. The minimum bactericidal concentrations (MBCs) were determined by identifying the lowest concentration

of the antibacterial agent that effectively eradicated bacteria within the designated time period, taking into account the specific experimental conditions.

3.5COMPOUND ISOLATION

A precisely measured 1 g sample of the crude *P. ostreatus* extract was placed in a 50-mL plastic tube with a screw-capped lid. Twenty millilitres of 70% methanol with 0.1% hydrochloric acid (v/v) were added, and the tube was vigorously shaken at 400 revolutions per minute for one night at room temperature. Subsequently, the mixture underwent centrifugation at 3000*x g* for 10 minutes. The residue underwent two additional rounds of extraction, each with 10 mL of the same solvent and under identical conditions. The collected supernatants were adjusted to a volume of 40 mL with the extraction solvent and filtered through a 0.45-µm polytetrafluoroethylene (PTFE) membrane filter. The resulting crude phenolic extract underwent hydrolysis with hydrochloric acid (final concentration of 2 N) and was heated at 85 °C for 1 hour. After cooling to room temperature, the samples were centrifuged at 3000 g for 5 minutes. The supernatant underwent a final filtration step. Phenolic acids and cinnamic acids were dissolved in distilled water, while flavonoids were dissolved in water containing 1% dimethyl sulfoxide (DMSO) and stored at -20 °C (Roy et al., 2022).

3.5.1 Isolation

For isolation, liquid-liquid extraction was employed to separate compounds based on their solubility in different solvents. Initially, the concentrated extract was

dissolved in a suitable solvent mixture. This solution was then partitioned with an immiscible solvent, such as ethyl acetate or chloroform, in a separating funnel. The partitioning process allowed the target phenolic compounds to distribute between the aqueous and organic phases based on their respective solubilities. After phase separation, the organic phase containing the phenolic compounds of interest was carefully separated and collected. The solvent was then evaporated under reduced pressure using a rotary evaporator to obtain a crude purified extract. This crude extract was further purified using techniques like column chromatography to isolate individual phenolic compounds. Liquid-liquid extraction provided an effective method to selectively extract and purify phenolic compounds from the initial extract, based on their differential solubility in solvents, thereby facilitating subsequent characterization and biological testing (Ecevit et al., 2022; Miklasińska-Majdanik et al., 2018).

Column chromatography was employed using a chromatography column packed with silica gel to separate compounds based on their polarity. Initially, the column was prepared by slurring silica gel with a suitable solvent to form a homogeneous slurry, which was then packed into the column under controlled pressure to ensure uniformity. The concentrated extract, dissolved in an appropriate solvent mixture, was carefully loaded onto the top of the chromatography column. As the solvent mixture (mobile phase) percolated through the column, compounds within the extract interacted differently with the silica gel (stationary phase) based on their

polarity. This differential interaction caused compounds to travel through the column at varying rates, thereby separating them into distinct fractions. Fractions containing the target phenolic compounds were collected sequentially as they eluted from the column. Each fraction was analysed using spectrophotometric methods to identify those containing the desired compounds. Subsequently, fractions of interest were pooled together and subjected to further isolation steps if necessary. As fractions eluted from the column, each containing a mixture of compounds, those showing similar spectrophotometric absorption patterns indicative of phenolic compounds were earmarked. Fractions with similar retention times on TLC plates or similar absorbance spectra in spectrophotometric analysis, corresponding to the presence of phenolic compounds, were pooled together. This pooling ensured that fractions containing the highest concentrations of the desired compounds were consolidated for further processing. Typically, fractions were pooled by combining them in a clean, solvent-compatible vessel. Care was taken to accurately measure and combine fractions to maintain the integrity of the pooled sample. Column chromatography provided a reliable method to purify phenolic compounds from the crude extract by exploiting differences in polarity, enabling isolation of individual compounds for subsequent characterization and biological evaluation.

3.6KINETIC GROWTH ASSAY

The kinetic growth assay was employed to monitor the proliferation of bacterial cultures over time, allowing for the assessment of their growth dynamics and response to various experimental conditions. In this assay, bacterial cultures were inoculated into a growth medium, and their growth was measured at regular intervals using techniques such as optical density measurement or colony counting. By tracking bacterial growth kinetics, researchers could evaluate the effects of different treatments, environmental factors, or genetic modifications on bacterial proliferation. This assay provided valuable insights into the physiological responses and adaptive mechanisms of bacterial populations, aiding in the study of microbial physiology, pathogenesis, and antimicrobial resistance. Individual compound analysis and dual combinations of compounds were conducted to assess their effects on bacterial growth kinetics using spectrophotometric analysis. Optical density at a wavelength of 595 nm (OD595) was measured at regular 30-minute intervals, maintaining a constant temperature of 37°C. Key parameters, including the specific growth rate (μ_{max}), representing the rate of optical density increase, the lag time (λ/h) indicating the period of adaptation before active growth, and the maximum population density at the stationary phase (Y_{max}), reflecting the highest optical density achieved when growth ceases, were determined. These parameters provide insights into the growth dynamics and response of bacterial cultures to the compounds and their combinations (Martins et al., 2017; Śliżewska & Chlebicz-Wójcik, 2020).

3.7SWISSADME

The structural characteristics and bioavailability of candidate compounds with potential antimicrobial properties were analyzed using the SwissADME software, developed by the Swiss Institute of Bioinformatics (SIB). Chemical structures of the compounds were inputted into the SwissADME platform via the official website (www.swissadme.ch). Compound structures were either drawn directly within the interface or uploaded in SDF, MOL, or PDB format. Upon submission, SwissADME performed a comprehensive analysis to evaluate diverse physicochemical properties critical for drug development. This included assessments of lipophilicity, solubility, pharmacokinetics, drug likeness, and medicinal chemistry parameters. Results from SwissADME provided detailed reports on each compound, presenting predictions such as LogP values for lipophilicity, aqueous solubility estimates, and ADME characteristics relevant to absorption, distribution, metabolism, and excretion profiles. The generated data facilitated insights into the molecular features and pharmacological profiles of the compounds under investigation. Researchers leveraged these insights to guide the design and optimization of novel antimicrobial agents, utilizing SwissADME as an essential tool in the early stages of drug discovery. The accessible nature of the platform and its ability to provide precise and actionable information proved instrumental in selecting promising compounds for further experimental validation and development processes (Asar et al., 2024).

3.8PROTEIN IDENTIFICATION

The focus of this research was on two key macromolecules: Penicillin-Binding Protein 2 (PBP2) and GAPDH from *Neisseria gonorrhoeae* strains. PBP2, crucial for cell membrane synthesis, was studied in two variants: one from the penicillin-resistant mutant strain FA6140 (PDB ID: 6HZJ) and the other from strain FA19 (PDB ID: 3EQU). These protein structures were obtained from the RCSB Protein Data Bank. Additionally, the crystallographic conformation of GAPDH, a key enzyme in glycolysis, derived from *Neisseria gonorrhoeae* strain NCCP11945, complexed with NAD (PDB ID: 5VMT), was investigated. The GAPDH macromolecule was also retrieved from the RCSB Protein Data Bank.

These macromolecules served as the basis for further structural and functional analysis in this research, providing insights into their roles in cellular processes and potential as therapeutic targets against drug-resistant *Neisseria gonorrhoeae* strains (Sarian et al., 2017).

3.8.1 Structure Validation

The structural integrity of both forms of Penicillin-Binding Protein 2 (PBP2) from N. is being assessed. The validation of gonorrhoeae strains FA6140 and FA19, along with GAPDH from strain NCCP11945, was thoroughly confirmed through a variety of analyses. The Procheck Structure Verification Methodology was employed to evaluate the predicted secondary structure and three-dimensional conformations of the proteins. This entailed constructing Ramachandran plots to

assess the bond angles (Phi and Psi angles) of each residue, thereby verifying the integrity of the protein's structural organisation. In addition, a G-factor metric was calculated to evaluate the probability of any inaccuracy or divergence in the protein's structure. The purpose of these validation techniques was to verify the precision and dependability of the protein structures acquired from the RCSB Protein Data Bank. By maintaining the proteins' structural integrity, future molecular docking and dynamics simulations with certainty, which will provide valuable insights into their interactions and functions can be undertaken (Vinholes et al., 2014).

3.9MOLECULAR DOCKING

3.9.1 Protein preparation

Prior to molecular docking simulations, the protein structures of Penicillin-Binding Protein 2 (PBP2) variants from *N. gonorrhoeae* strains FA6140 and FA19, as well as GAPDH from strain NCCP11945, underwent meticulous preparation. The protein complexes retrieved from the RCSB Protein Data Bank were processed using Discovery Studio 2021 to remove heteroatoms and optimize their structures for docking purposes. This involved modelling the protein structures and ensuring their readiness for subsequent docking analyses. After preparation, the protein structures were saved in .pdb format, ready for further ligand-protein docking simulations (Abdelrheem et al., 2020). This initial

step of protein preparation ensured that the proteins were in suitable conformations for accurate molecular interactions with the ligands of interest.

3.9.2 Ligand preparation

For ligand preparation, target ligands in 3D .sdf format with favourable drug-likeness scores were sourced from the PubChem database. These ligands, namely (IUPAC Name: 3,4-dihydroxybenzoic acid), *p*-coumaric acid (IUPAC Name: (E)-3-(4-hydroxyphenyl)pro*p*-2-enoic acid), ferulic acid (IUPAC Name: (E)-3-(4-hydroxy-3-methoxyphenyl)pro*p*-2-enoic acid), quercetin (IUPAC Name: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one), and rutin (IUPAC Name: 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one), were selected based on their potential pharmacological relevance and chemical properties. The ligands were retrieved in .sdf format and then converted to .pdb format to ensure compatibility with the subsequent docking simulations. This process involved preparing the ligands in a suitable molecular format for interaction with the protein targets. Once converted, the ligands were ready for docking studies, where their interactions with the protein targets could be evaluated to assess their binding affinities and potential as antimicrobial agents.

3.9.3 Ligand-protein docking

Ligand-protein docking was executed using both PyRx and Schrödinger software to ensure a comprehensive analysis of binding interactions. Initially, PyRx software was employed to facilitate the ligand-protein binding procedure utilizing the AutoDock Vina plugin. This approach allowed for the efficient screening of ligands against the target proteins, Penicillin-Binding Protein 2 (PBP2) from *N. gonorrhoeae* strains FA6140 and FA19, as well as GAPDH from *N. gonorrhoeae* NCCP11945. Post-docking, the intermolecular associations between the proteins and ligands were evaluated based on their binding energies, providing a quantitative measure of the interaction strength. To gain further insights into the conformations of the docked ligands, detailed analyses were conducted using Discovery Studio 2021, which allowed for the visualization and assessment of the binding poses and interactions within the active sites of the target proteins.

Simultaneously, the Schrödinger suite was employed to perform additional docking studies, ensuring the robustness and reliability of the results. This dual approach enabled the validation and comparison of docking outcomes, thereby enhancing the accuracy of the binding affinity predictions. The integration of these advanced computational tools facilitated a thorough investigation of the potential of the selected ligands as inhibitors of drug-resistant *N. gonorrhoeae* strains, contributing to the development of novel antimicrobial agents.

3.10 MOLECULAR DYNAMICS (MD) SIMULATIONS

Molecular dynamics (MD) simulations were conducted to further investigate the stability and dynamics of the protein-ligand complexes identified from docking studies. For each investigated system, two independent replicates of the MD simulations were performed to account for the intrinsic variability in molecular systems and to ensure the robustness of the results. The compounds selected for MD simulations were based on their docking scores, with priority given to those exhibiting the highest binding affinities and most favourable interaction profiles with the target proteins. These compounds included protocatechuic acid, *p*-coumaric acid, ferulic acid, quercetin, and rutin, all of which showed significant potential in the docking studies against Penicillin-Binding Protein 2 (PBP2) and GAPDH from *N. gonorrhoeae*.

MD simulations were carried out using the Desmond package, which allows for detailed and accurate modelling of molecular interactions over time. The simulations were initiated by equilibrating the systems under physiological conditions, ensuring that the proteins and ligands adopted stable conformations before proceeding with the production runs. Each replicate simulation was initiated with unique random seed values for the initial velocities of atoms, introducing diversity in the trajectory sampling and enhancing the reliability of the observed results. Throughout the MD simulations, various parameters such as root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), and

binding free energy were monitored to assess the stability and binding affinity of the ligands to the target proteins. The RMSD profiles were analysed to ensure that the systems reached equilibrium, and consistent patterns across replicates indicated reliable simulation outcomes. Binding free energy calculations provided insights into the strength and nature of the interactions, with lower energies indicating stronger and more stable binding.

The data from the MD simulations were analysed independently for each replicate, allowing for the computation of mean binding affinities and residence times. This approach ensured that the results were statistically significant and not biased by initial conditions or random fluctuations. By performing multiple replicates, the study achieved a higher level of confidence in the observed interactions and provided a comprehensive understanding of the dynamic behaviour of the protein-ligand complexes.

The findings from the MD simulations were crucial in validating the initial docking results and in identifying potential lead compounds for further development. The consistent binding events and similar RMSD patterns observed across replicates confirmed the reliability of the selected ligands as effective inhibitors of drug-resistant *N. gonorrhoeae*. This comprehensive approach, combining docking studies with rigorous MD simulations, significantly contributed to the identification of promising candidates for novel antimicrobial agents.

3.11 NORMAL MODE ANALYSIS

Normal Mode Analysis (NMA) was employed to further elucidate the dynamic properties and intrinsic flexibility of the protein-ligand complexes identified from the docking and MD simulation studies. NMA provides a comprehensive understanding of the collective motions within a protein structure, which are essential for its biological function and interaction with ligands. The NMA was performed on the Penicillin-Binding Protein 2 (PBP2) from both *N. gonorrhoeae* strain FA6140 and FA19, as well as on the GAPDH from *N. gonorrhoeae* NCCP11945. These analyses were conducted using the Normal Mode Wizard (NMWiz) in VMD (Visual Molecular Dynamics) and ProDy software packages, which are well-suited for detailed normal mode calculations and visualization of protein dynamics.

Initially, the minimized structures of the protein-ligand complexes obtained from the MD simulations were used as input for the NMA. This step ensured that the structures were in a low-energy state, representing biologically relevant conformations. The NMA involved computing the Hessian matrix of the second derivatives of the potential energy with respect to the atomic coordinates, which describes the curvature of the energy landscape around the equilibrium structure. The lowest frequency normal modes, typically associated with large-scale conformational changes, were analysed to gain insights into the intrinsic flexibility and potential functional motions of the proteins. These modes are

particularly relevant as they often correspond to biologically significant movements such as domain motions, hinge-bending, and allosteric transitions. For each protein-ligand complex, the first few non-trivial normal modes were visualized to identify the predominant motions. The amplitude and direction of these motions provided valuable information on how the binding of ligands might influence the dynamic behaviour of the proteins. Additionally, the overlap between the normal modes and the conformational changes observed during the MD simulations was assessed to ensure consistency and to validate the relevance of the identified modes.

The results from the NMA highlighted regions of the proteins that exhibited significant flexibility, which could be critical for their function and interaction with ligands. For PBP2, the analysis revealed key movements in the active site region that might facilitate the binding of inhibitors. Similarly, for GAPDH, the normal modes indicated conformational changes that could impact its catalytic activity and interaction with glycolytic intermediates.

Overall, the NMA provided a deeper understanding of the dynamic properties of the protein-ligand complexes and complemented the findings from the MD simulations. This integrated approach allowed for a more comprehensive characterization of the potential impact of the ligands on protein function and stability, thereby aiding in the identification and optimization of novel antimicrobial agents against drug-resistant *N. gonorrhoeae*.

3.12 STATISTICAL ANALYSIS

Statistical parameters were computed using the JMP Pro 16 software package. This analysis allowed for the application of various statistical methods to evaluate the data, including descriptive statistics, hypothesis testing, and regression analysis. The software facilitated comprehensive data exploration and interpretation, contributing to the robustness of the statistical analysis conducted in the study. The study employs a range of statistical parameters to evaluate data and draw meaningful conclusions. One key parameter is the p-value (probability > ChiSq), which indicates the probability of obtaining a test statistic as extreme as the one observed under the null hypothesis. The chi-square statistic, accompanied by degrees of freedom, is used to compare observed data with expected data. The coefficient of determination (R^2) measures the proportion of variability in the dependent variable that can be explained by the independent variable(s), while the root mean square error (RMSE) represents the average deviation between observed values and those predicted by the model. The regression equation, including its slope and intercept, defines the relationship between independent and dependent variables. Confidence intervals provide a range within which the true value of a population parameter is expected to lie, indicating precision.

Further, the study uses log likelihood values to assess the fit of statistical models to observed data. Estimated probabilities are calculated to understand the

likelihood of specific outcomes or parameter estimates. Descriptive statistics such as mean, standard deviation, and variance are employed to summarize data distribution and variability. In molecular dynamics simulations, B-factors indicate the flexibility of specific residues, while root mean square fluctuation (RMSF) and root mean square deviation (RMSD) measure deviations over time from reference positions. Eigenvalues from normal mode analysis reflect the stiffness of modes in structural dynamics, and the covariance matrix captures interactions between different residues or atoms. In molecular docking studies, binding affinity assesses the strength of interactions between ligands and target proteins. By integrating these statistical parameters, the study aims to thoroughly understand antimicrobial resistance patterns, molecular interactions, and potential therapeutic strategies for *Neisseria gonorrhoeae* isolates.

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CHAPTER 4: IDENTIFICATION OF EMERGING MULTIDRUG-RESISTANT *NEISSERIA GONORRHOEAE* ISOLATES AGAINST FIVE MAJOR ANTIMICROBIAL AGENT OPTIONS

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Preface

This article reported on the detection and analysis of multidrug-resistant strains of *Neisseria gonorrhoeae* isolates taken in KwaZulu Natal that were used in this project. This article was published in the Medical Sciences (MDPI) Journal on March 31, 2023, in Volume 11, Issue 2. DOI link: https://doi.org/10.3390/medsci11020028

4.1ABSTRACT

Antimicrobial drug resistance in *Neisseria gonorrhoeae* has been extensively documented worldwide. Unfortunately, the situation in Sub-Saharan Africa has not received much attention. Establishing diagnostics and expanding surveillance is crucial in order to prevent the emergence of illnesses that can resist multiple treatments. It is crucial to monitor antimicrobial susceptibility in order to collect data that can be used to develop treatment recommendations that will lead to effective therapy, a reduction in complications and transmission of gonorrhoeae, and successful treatment. Government authorities can establish research and prevention goals, along with treatment suggestions, by utilising data from the Gonococcal Antimicrobial Surveillance Program (GISP). Local and state health authorities rely on GISP data to inform decisions regarding the distribution of STI prevention services and resources, to facilitate proactive planning, and to share information on effective treatment practices. Through the utilisation of molecular and culture techniques, an investigation was conducted to examine the prevalence of antibiotic resistance in isolates from KwaZulu Natal, South Africa. A significant number of gonococcal isolates (48%) demonstrated complete resistance to ciprofloxacin, while penicillin and tetracycline resistance rates were both 14%. Out of all the gonococcal isolates that were tested, it was found that only one of them displayed resistance to azithromycin. The minimum inhibitory concentration (MIC) for this isolate was measured to be 1.5 µg/mL. All gonococcal isolates tested were susceptible to the effectiveness of ceftriaxone.

4.2INTRODUCTION

In 2016, the World Health Organisation (WHO) estimated that there were 87 million new cases of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) infections among individuals aged 15 to 49 (World Health Organization, 2016). Sub-Saharan Africa was found to have the highest prevalence of *N. gonorrhoeae* (Yakobi & Pooe, 2022). With the rise of resistance to antimicrobial drugs, *N. gonorrhoeae* has become a major global public health concern. It is listed on the WHO global priority list of antibiotic-resistant bacteria, highlighting its significance (Pérez-Gracia & Suay-García, 2021; Suay-García & Pérez-Gracia, 2018; Unemo, 2015). There is growing concern over the emergence of ceftriaxone and azithromycin-resistant strains of *N. gonorrhoeae* in Australia and the United Kingdom, as recent studies have shown (Chen et al., 2019; Jacobsson et al., 2021; Suay-García & Pérez-Gracia, 2018). In addition, there is a significant knowledge gap when it comes to understanding the antibiotic resistance spectrum of *N. gonorrhoeae* strains in Sub-Saharan Africa, which happens to have the highest infection frequency. This lack of knowledge raises serious concerns about the potential spread of this incurable *N. gonorrhoeae* strain (Maduna et al., 2020). One of the main challenges in Sub-Saharan Africa is the limited availability of laboratory diagnostic facilities, which makes it difficult to gather accurate data on antibiotic resistance in sexually transmitted infections (STIs). Additionally, the reliance on syndromic care further contributes to the scarcity of this information. There are several drawbacks associated with syndromic management. These include the

absence of susceptibility testing, the difficulty in detecting silent infections, limited options for comprehensive monitoring, and a lack of data on patients who have not responded to therapy (Kharsany & Karim, 2016; Vrioni et al., 2018). In South Africa, 1 g of azithromycin and 250 mg of ceftriaxone are often used to treat syndrome-related diseases (Kularatne et al., 2018). Research has shown that certain groups, such as men who have sex with other men (MSM) and individuals with recurring episodes of *N. gonorrhoeae*, play a significant role in the spread of treatment resistance (Maduna et al., 2020). Multiple studies have shown that MSM in South Africa and other African countries face a significant challenge with *N. gonorrhoeae* infections. The MSM community has been documented as the source of the first two cases of cefixime-resistant *N. gonorrhoeae* infections in Africa (Maduna et al., 2020; Peters & Maduna, 2020). It is crucial to have a comprehensive understanding of the antibiotic resistance profile of gonococcal populations in core transmission groups and those under sentinel monitoring. This knowledge plays a vital role in shaping clinical treatment recommendations and policy planning (Lewis, 2011; Yakobi & Pooe, 2022). Surveillance plays a vital role in the detection and tracking of gonococcal resistance. In many clinical settings, the availability of *N. gonorrhoeae* culture is limited (Kirkcaldy, 2019). Diagnostic procedures that do not rely on culture, such as nucleic acid amplification tests (NAATs), are becoming more common in many countries. However, antibiotic susceptibility testing is not commonly performed in most laboratories (Bodie et al., 2019; Meyer & Buder, 2020). In these contexts, *N.*

gonorrhoeae infections are not typically accompanied by information on drug susceptibility, and alternative monitoring methods are employed. As a result, the Centres for Disease Control and Prevention (CDC) conducted annual extensive investigations to assess the antibiotic resistance of *N. gonorrhoeae*, like the National Gonorrhoeae Therapy Monitoring Study (Kirkcaldy, 2019; Kularatne et al., 2018). This research aims to provide a comprehensive analysis of the resistance patterns and trends of *N. gonorrhoeae* to specific antimicrobials in isolates obtained from the University of KwaZulu Natal.

4.3MATERIAL AND METHODS

Please refer to chapter 3, subsection 3.1, 3.2.1 and 3.12, page 92 – 93 and 117 – 118, respectively, for a detailed material and methods protocol.

4.4RESULTS

4.4.1 Identification of target isolates

We obtained a collection of sixty-four *Neisseria gonorrhoeae* isolates from urethral swabs, courtesy of the University of KwaZulu Natal's Medical Microbiology department. The isolates were cultured on chocolate agar and stored at -80 °C for preservation. Afterwards, the isolates were placed on New York City (NYC) agar and left to incubate at 37 °C in a 5% CO_2 atmosphere for 24 hours. After the incubation period, the isolates were subjected to a set of confirmatory tests. These tests included Gram-staining, which revealed the presence of gram-negative diplococci. Additionally, the isolates tested positive

for oxidase and catalase. All the isolates have been confirmed to be *N. gonorrhoeae*. In order to preserve the pure culture isolates for future analyses, they were suspended in trypticase soy broth with 20% glycerol and immediately frozen at -80 °C. The meticulous preparation and thorough confirmation process ensured the utmost reliability of the isolates for further investigation into their phenotypic and genotypic characteristics, specifically regarding their antimicrobial resistance profiles.

4.4.2 Antimicrobial Susceptibility Testing

All the isolates analysed showed resistance to multiple antibiotics, indicating that they are classified as multidrug-resistant *N. gonorrhoeae*. The antimicrobial susceptibility data related to resistance in *N. gonorrhoeae* are displayed in Table 4.1. The majority of the isolates displayed resistance to ciprofloxacin, with a staggering 94% (n = 60) being phenotypically resistant. Penicillin resistance was observed in 45% (n = 29) of the isolates, while tetracycline resistance was found in 44% (n = 28). Resistance to azithromycin was found in 2% of gonococcal isolates, with a MIC of 1.5 µg/mL, as determined by interpreting ECOFF values. The isolates did not show any resistance or decreased susceptibility towards ceftriaxone. Out of all the isolates, a mere 4.7% showed susceptibility to all the target antibiotics. On the other hand, a significant 20.3% of the isolates displayed resistance to at least one of the target antibiotics.

Table 4.1: Antimicrobial susceptibility profiles and MICs for the *Neisseria gonorrhoeae* isolates (n = 64).

	Number of Isolates		**MIC (µg/mL)**		
Drug	**Susceptible**	**Resistant**	**Median**	**Mean**	**Range**
[a] Ciprofloxacin	4	60	2	8	0.016–32
[b] Azithromycin	63	1	0.094	0.2	0.016–1.5
[a] Penicillin	35	29	0.094	3.7	0.016–32
[a] Tetracycline	36	28	0.094	3.1	0.016–32
[a] Ceftriaxone	64	0	0.006	0.012	0.002–0.12

[a]EUCAST breakpoints were used to classify strains as susceptible or resistant.

[b]ECOFF value used classify strains as susceptible or resistant.

The majority of these resistant isolates (92%) were found to be resistant to ciprofloxacin, while the remaining 8% showed resistance to penicillin. Out of all the *N. gonorrhoeae* isolates, a significant number, specifically 26 isolates (41%), displayed resistance to two of the antibiotics being studied. All 26 of these isolates showed a high level of resistance to ciprofloxacin, with every single one being resistant. Out of these, 12 isolates (46.2%) also displayed resistance to penicillin, while 14 isolates (53.8%) showed resistance to tetracycline. The majority of c*o*-resistance in these clinical isolates was found against tetracycline and ciprofloxacin. 21 isolates displayed resistance to three target antibiotics, specifically ciprofloxacin, tetracycline, and penicillin. Out of all the isolates

tested, only ISID 26 demonstrated an impressive ability to resist four different antibiotics: ciprofloxacin, tetracycline, penicillin, and azithromycin.

In this study, it was discovered that there were a total of 62 cases where the clinical isolates showed complete resistance to the antibiotics being tested. Out of the 29 instances of penicillin resistance, 31% were found to be resistant. Similarly, 39% of the 28 instances of tetracycline resistance showed resistance, while a staggering 53% of the 60 instances of ciprofloxacin resistance were found to be resistant. Out of all the isolates tested, only a small number showed complete resistance to the three antibiotic drugs (ISID 5, 7, 21, 45, 59). Additionally, a few isolates demonstrated complete resistance to tetracycline and ciprofloxacin, while a small percentage of isolates showed complete resistance to penicillin and ciprofloxacin. However, a small percentage of isolates (6%) showed complete resistance to tetracycline and penicillin. The isolate demonstrated complete resistance to ciprofloxacin and tetracycline, while penicillin resistance was observed at a MIC of 8 μg/mL.

The relationship between penicillin-resistance and tetracycline-resistance development in these clinical isolates showed a strong covariance value of 60.6 and a moderate correlation value of 0.43. The lower 95% confidence interval (CI) was 0.2, the upper 95% CI was 0.6, and the *p*-value was 0.0004, indicating statistical significance. The average penicillin resistance was measured at 7.7 g/mL, with a standard deviation (SD) of 11.8. Similarly, the average tetracycline

resistance was found to be 8.1 g/mL, with an SD of 11.9. The mean value was found to be 7.7, with a standard error (std error) of 1.4. Based on the analysis, the RSquared value (the coefficient of determination) was found to be 0.19. However, the lack of fit had an F-Ratio of 0.74 and the probability > F was calculated to be 0.72. The parameter estimates for the intercept were observed at 4.2, with a standard error of 4.2. In the analysis, the t-value to assess the disparity between the means of antibiotic resistance. The resulting t-ratio was 2.6, indicating a probability greater than t of 0.012 (refer to Figure 4.1), was used. Through a bivariate fit analysis, it was discovered that a strong correlation exists between two variables, suggesting a potential connection between the development of penicillin and tetracycline resistance. The relationship between these variables is particularly pronounced, indicating a significant linear relationship.

No statistical significance was found in the relationship associations between the emergences of resistance in clinical isolates of *N. gonorrhoeae*, as indicated by the *p*-values. Based on the statistical analysis, the covariance between penicillin and ciprofloxacin resistance was 32.8, indicating a potential relationship between the two. Additionally, the correlation coefficient of 0.2 suggests a weak positive correlation between the two variables. The average level of ciprofloxacin resistance was 19.6 µg/mL, with a standard deviation of 14.1. The fit had a low R-Squared value of 0.03, indicating that it did not explain much of the variability

in the data. The lack of fit was also evident from the F-Ratio of 0.3 and the probability > F value of 0.12. The intercept's parameter estimations were 4.5 with a standard error of 2.5. When analysing the disparity in antibiotic resistance, the t-value. the findings revealed a t-ratio of 1.8, accompanied by a probability greater than t of 0.082 was employed. There is a strong association between resistance to tetracycline and ciprofloxacin, as indicated by a covariance value of 21.8 and a correlation value of 0.13. The 95% confidence interval ranged from 0.11 to 0.36. The fit had a low R-Squared value of 0.02, indicating a weak relationship. On the other hand, the lack of fit showed an F-Ratio of 1.8 and a probability > F of 0.07. Based on the given data, the parameter estimates for the intercept were 18.4, with a 2.1 standard error. After conducting the analysis, a t-ratio of 8.6, which indicates a significant difference in antibiotic-resistance means was obtained. The probability of this result occurring by chance alone is less than 0.001.

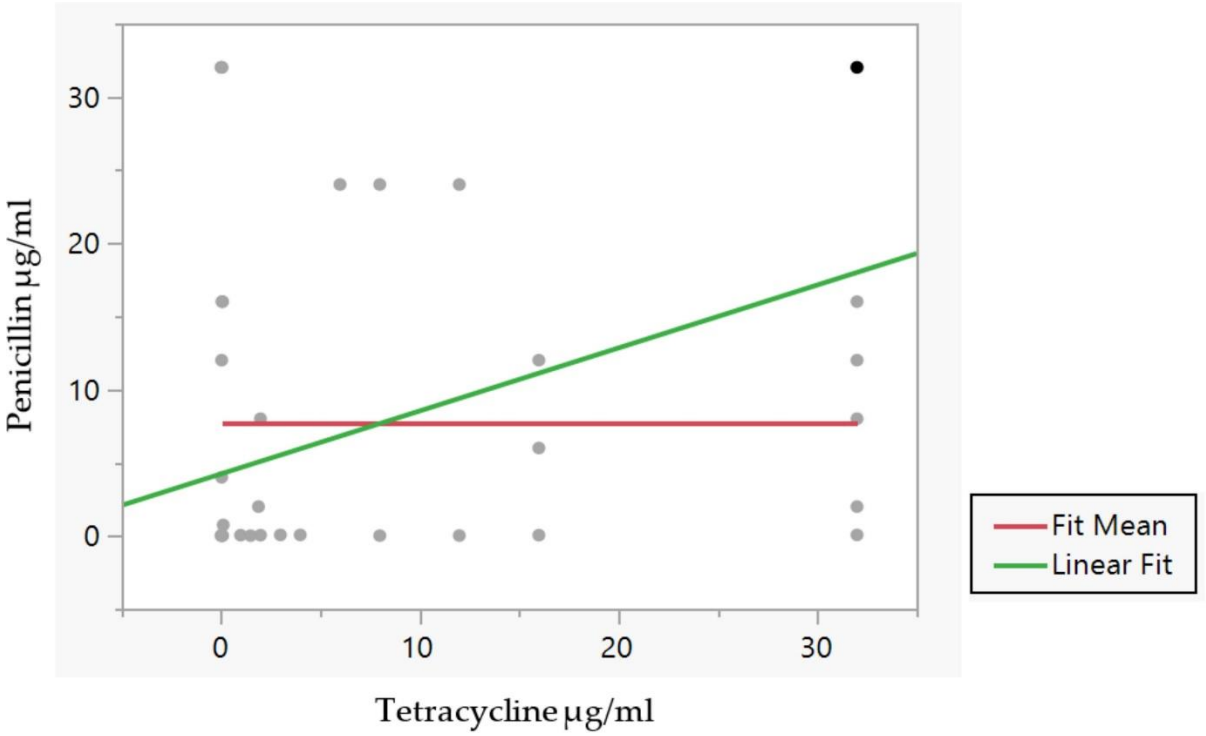

Figure 4.1: An analysis on 64 clinical isolates of *N. gonorrhoeae,* examining the relationship between penicillin resistance and tetracycline resistance. Each point on the graph represents the antibiotic concentration required to effectively inhibit isolates that exhibit resistance to both penicillin and tetracycline.

When analysing two of the three antibiotic resistances (penicillin, tetracycline, and ciprofloxacin), it is observed that these variables exhibit a positive correlation and move in the same direction. The data that has been presented indicates an increase in the covariance values, suggesting a significant finding in this regard. Correlation coefficients were utilised to assess the strength of the linear relationship between two variables at a specific point in time. With the help of a correlation coefficient interval chart, the relationship between antibiotic resistance and its direction and degree was determined. In this study, some

interesting correlations among the isolates were observed. Firstly, there was a very weak correlation of 0.13 between tetracycline resistance and ciprofloxacin resistance. Secondly, a weak positive correlation between penicillin resistance and ciprofloxacin resistance (see Figure 4.2) was found. Lastly, a moderately positive correlation between penicillin resistance and tetracycline resistance (refer to Figure 4.1) was observed. These findings shed light on the relationships between different types of resistance among the isolates.

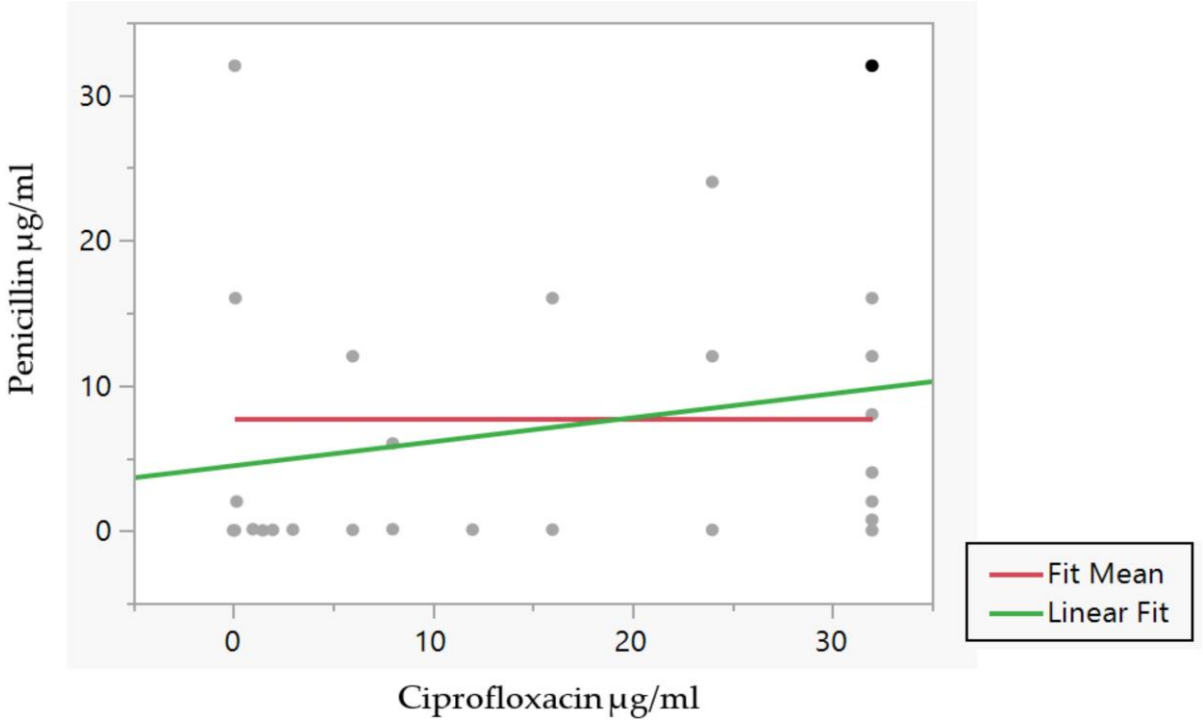

Figure 4.2: An analysis on 64 clinical isolates of *N. gonorrhoeae*, examining the relationship between penicillin resistance and ciprofloxacin resistance. Each point on the graph represents the antibiotic concentration required to effectively inhibit isolates that exhibit resistance to both penicillin and ciprofloxacin.

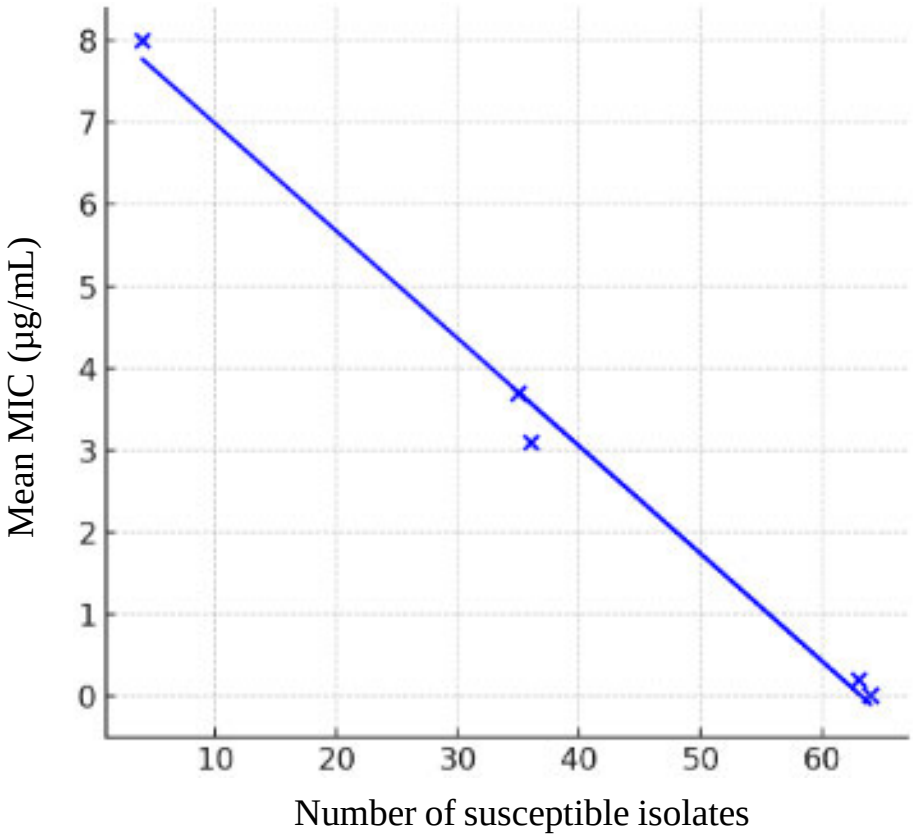

Figure 4.3: Susceptible vs. Mean MIC (correlation:-1:00)

The linear plots with regression lines provide a clear visualization of the relationships between the number of susceptible/resistant isolates and the Mean MIC values. Figure 4.3 presents a scatter plot with a linear regression line shows a strong negative correlation. As the number of susceptible isolates increases, the Mean MIC values decrease, with a correlation coefficient of -0.996, which confirms the very strong negative correlation.

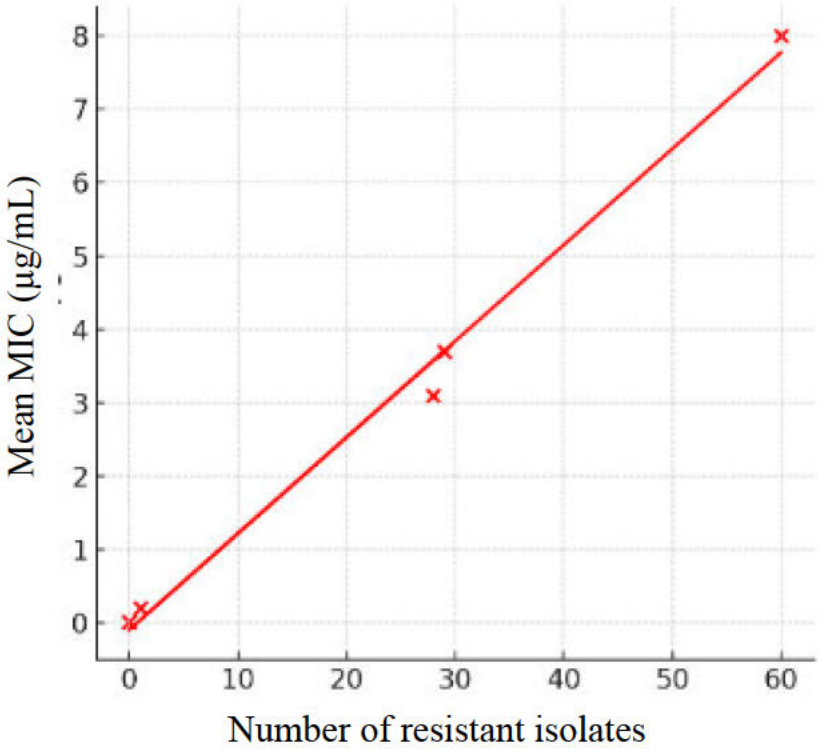

Figure 4.4: Resistance vs. Mean MIC (correlation: 1:00)

Figure 4.4 presents a scatter plot with a linear regression line shows a strong positive correlation. As the number of resistant isolates increases, the Mean MIC values increase. The correlation coefficient was found to be 0.996, which confirms the very strong positive correlation. These linear plots with regression lines reinforce the findings from the correlation coefficients. They clearly illustrate the inverse relationship between the number of susceptible isolates and the Mean MIC values and the direct relationship between the number of resistant isolates and the Mean MIC values. These insights are crucial for understanding the antimicrobial resistance patterns of *N. gonorrhoeae* and can guide treatment strategies.

4.5DISCUSSION

Antimicrobial resistance in *N. gonorrhoeae* is a pressing concern for global health as it raises the risk of *N. gonorrhoeae* becoming untreatable (Semchenko & Seib, 2021). It is evident from the findings that there is a need for increased focus on *N. gonorrhoeae* infections, especially in low-resource settings like the South African public health care system. All the strains of *N. gonorrhoeae* that were studied were classified as multidrug-resistant bacteria. Most of the isolates exhibited significant levels of resistance to ciprofloxacin, followed by tetracycline, penicillin, and azithromycin, in that order. In South Africa, the implementation of syndromic treatment for STIs has allowed for the extended use of these medications (Kularatne et al., 2018; Torrone et al., 2018). These target drugs have shown significant rates of antimicrobial resistance, which aligns with data from national surveillance and recent studies conducted in the South African province of Gauteng (Ranmini Kularatne et al., 2018). The research findings (with a 94% resistance rate) were consistent with these trends. Recently, there has been a concerning increase in resistance rates for Ciprofloxacin, which has led to its removal from the market (Fourie et al., 2021). It is conceivable that this is attributable to the use of ciprofloxacin in the treatment of male dysuria (Morris et al., 2019), along with the ongoing use of ciprofloxacin by specific medical centres for the treatment of male urethral discharge. Currently, repurposing this medicine for syndromic disease treatment is not feasible due to the consistently high resistance rate mentioned in recent studies (Hummell & Kirienko, 2020; Lin et

al., 2021). Researchers found that mutations leading to resistance to ciprofloxacin developed gradually in specific regions of the *gyrA* and *parC* genes, which are responsible for quinolone resistance (Hamasuna et al., 2018). According to Kivata et al., 2020, the increased prevalence of tetracycline resistance is believed to be linked to the administration of doxycycline as a component of syndromic therapy for non-gonococcal urethritis. Most tetracycline-resistant isolates commonly contain the *tetM* plasmid and RpsJ V57M polymorphisms. In addition, various isolates have demonstrated the presence of the GGI, a type 4 secretion system associated with the spread of antimicrobial resistance to multiple antimicrobials within gonococcal species, as well as plasmid-mediated antimicrobial resistance (Cristillo et al., 2019). A significant majority of gonococcal strains possess both GGI and plasmid-mediated antimicrobial resistance in their genomes (Kivata et al., 2020). Until 2015, azithromycin was not commonly used to treat urethral discharge in South Africa due to limited availability in the public health system. Although the use of azithromycin for treating STIs is relatively recent, studies conducted in South Africa revealed that 15% of MSM isolates showed resistance to azithromycin (Maduna et al., 2020; Rambaran et al., 2019). Out of the 64 isolates that were tested, only one showed resistance to this antibiotic. The resistance was classified as "resistant" based on the EUCAST breakpoint, with a minimum inhibitory concentration (MIC) of 1.5 g/mL. Due to its common use in combination with other medications, the determination of azithromycin susceptibility breakpoint is no longer provided by

EUCAST. Instead, azithromycin resistance is defined using ECOFFs. It is important to consider the inclusion of male groups, particularly MSM, in routine drug screening. Currently, there is uncertainty regarding whether the resistance to azithromycin is confined to specific groups or if it has spread to the wider population in South Africa. Among symptomatic individuals at sentinel sites, national surveillance revealed a moderate occurrence (3%) of azithromycin resistance in N. gonorrhoeae. Unfortunately, this monitoring does not specifically address important transmission groups. Contrary to expectations, recent findings from two clinics in the KwaZulu Natal area reveal a high prevalence of azithromycin resistance. According to the studies conducted by Kularatne et al., 2018 and Maduna et al., 2020, approximately 68% of isolates exhibited resistance to azithromycin. In this investigation, agar dilution methods were used to measure the MIC instead of the E-test. However, the collective findings from these studies strongly support the importance of closely monitoring azithromycin resistance. This is because the newly established resistance can hinder the effectiveness of syndromic treatment. Many countries now recommend ceftriaxone as the primary treatment for *N. gonorrhoeae*. While the combined therapy of azithromycin and ceftriaxone has been recommended by WHO, it may be necessary to re-evaluate this treatment approach for *N. gonorrhoeae*. When it comes to syndromic treatment, it is recommended to use a combination of azithromycin and ceftriaxone. Studies have shown that this dual therapy can effectively surpass resistance thresholds and ensure adequate levels of macrolide consumption

(Shigemura et al., 2015; Terkelsen et al., 2017). It is promising to note that none of the isolates showed resistance to ceftriaxone. While the study found that azithromycin and ceftriaxone resistance was not significant, it is important to highlight the growing concern of antimicrobial resistance in N. gonorrhoeae. Specifically, the resistance to ceftriaxone, which is currently the first-line monotherapy, poses a serious threat to effective gonorrhoeae control in China. Between 2013 and 2016, data collected by the China Gonococcal Antimicrobial Resistance Surveillance Program (China-GRSP) showed a significant occurrence (ranging from 9.7% to 12.2%) of 3827 isolates with decreased susceptibility to ceftriaxone (MIC 0.125 mg/L) (S. C. Chen et al., 2020). Since then, it has been recorded in Japan, Australia, Canada, Denmark, Ireland, the United Kingdom, and Singapore (Eyre et al., 2019; Golparian et al., 2018; Ko et al., 2019; Lahra et al., 2018; Lefebvre et al., 2018; Nakayama et al., 2016; Terkelsen et al., 2017). Shigemura and his colleagues also found that a deletion mutation in the mtrR promoter region could be a likely cause of azithromycin resistance and might strongly indicate higher MICs (0.5 g/mL or higher) in *N. gonorrhoeae* infection. According to a recent study, it was discovered that gonococci showed decreased sensitivity to azithromycin, potentially compromising the effectiveness of the drug. It has been noted that the decreased susceptibility to azithromycin can be attributed to a range of different processes. Antibiotic resistance has emerged as a consequence of the extensive use of these medications in both medical practices and animal farming.

Given the anticipated outbreak of MDR *N. gonorrhoeae*, it is of utmost importance to enhance sexual health care services nationwide. To prevent the emergence of an untreatable epidemic of *N. gonorrhoeae* in Sub-Saharan Africa, it is crucial to implement strong clinical governance and antimicrobial stewardship practices. Additionally, the use of molecular diagnostics, careful selection of treatment regimens, evaluation of new drugs like zoliflodacin, and investment in an improved antimicrobial surveillance system are all necessary measures. This study has a few limitations, with one of the most significant being the limited number of isolates that were assessed. Additionally, there is a difficulty in understanding high-risk groups and core transmission groups because of a scarcity of patient information. Although the number of isolates studied was limited, the findings offer a glimpse into the antibiotic resistance of *N. gonorrhoeae* strains in KwaZulu Natal. These results align with other published reports. In order to gain a deeper understanding of the resistance mechanism exhibited by the 64 isolates under investigation, we will conduct whole genome sequencing.

The antimicrobial resistance patterns in *N. gonorrhoeae*, analysing correlations between the number of susceptible or resistant isolates and MIC values for various antibiotics. For ciprofloxacin, there were 4 susceptible isolates and 60 resistant isolates, with MIC values showing a median of 2 µg/mL, mean of 8 µg/mL, and range from 0.016 to 32 µg/mL. The analysis indicated a positive

correlation between the number of resistant isolates and higher MIC values, suggesting increased concentrations of ciprofloxacin required to inhibit growth as resistance prevalence rises. Clinically, elevated MIC values indicate reduced effectiveness, posing challenges for treatment efficacy. Similar detailed correlation analyses are required for azithromycin, penicillin, and tetracycline, revealing varied patterns where some drugs exhibit stronger positive correlations between resistant isolates and high MIC values compared to others. Understanding these correlations informs treatment strategies, guiding clinicians towards effective therapeutic choices amidst rising resistance. For ceftriaxone, with 64 susceptible isolates and no resistant isolates detected, MIC values showed a median of 0.006 µg/mL, mean of 0.012 µg/mL, and range from 0.002 to 0.12 µg/mL, indicating high effectiveness against susceptible strains. Consequently, ceftriaxone remains a recommended treatment option due to its very low MIC values and absence of resistance. Overall, the thesis underscores significant correlations observed across multiple antibiotics, linking the prevalence of resistant isolates to MIC values. These findings highlight the critical need for monitoring resistance patterns and adapting treatment guidelines accordingly. Insights into these correlations inform public health strategies, supporting antibiotic stewardship efforts and the development of effective treatment protocols to combat antimicrobial resistance in *N. gonorrhoeae*. Ultimately, the thesis emphasizes how such analyses provide essential insights into antimicrobial

resistance dynamics, shaping clinical practices and public health policies to address this pressing issue effectively.

4.6CONCLUSION

Monitoring antibiotic susceptibility of *N. gonorrhoeae* can enhance understanding of gonococcal resistance, shape policy and preventive measures, and offer data for regular updates to national treatment recommendations. An investigation has identified the emergence of azithromycin resistance in *N. gonorrhoeae*, which could potentially complicate its treatment in the future. This information can be utilised by local and national health agencies to make informed decisions regarding the allocation of services and resources for the prevention of sexually transmitted infections (STIs). It can also play a crucial role in shaping the planning of preventive and control initiatives. Continued monitoring, appropriate treatment, the development of new medications, and prevention of transmission are crucial strategies for reducing the occurrence and impact of *N. gonorrhoeae*.

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CHAPTER 5: A MOLECULAR AND PHENOTYPIC ANALYSIS OF THE PREVALENCE AND PATTERNS OF ANTIMICROBIAL RESISTANCE IN *NEISSERIA GONORRHOEAE* ISOLATES FROM KWAZULU NATAL, SOUTH AFRICA

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Preface

This study aimed to shed light on the pressing issue of antimicrobial resistance in the target *Neisseria gonorrhoeae* isolates identified in this study. Through both molecular and phenotypic analyses, the resistance patterns and identified gaps in strategies for effective management and control of gonorrhoeae were investigated. This investigation was published with the Scientific Africa (Elsevier) on August 15, 2024, in Volume 25. DOI link: https://doi.org/10.1016/j.sciaf.2024.e02334.

5.1ABSTRACT

Antimicrobial drug resistance in *Neisseria gonorrhoeae* has been widely documented globally, but the situation in Sub-Saharan Africa has not received much attention. Establishing diagnostics and expanding surveillance is crucial in order to prevent the emergence of illnesses that are resistant to multiple treatments. Monitoring antimicrobial susceptibility is essential for collecting data that can guide treatment recommendations, leading to successful therapy, decreased complications and transmission of gonorrhoea, and efficient treatment. Government authorities can establish research and prevention goals, as well as treatment guidelines, by utilising data from the Gonococcal Antimicrobial Surveillance Program (GISP). Local and state health authorities can utilise GISP data to inform their decisions regarding the distribution of STI prevention services and resources, as well as to assist in developing effective preventive strategies. Additionally, this data can be used to share information on the most effective treatment practices. An investigation was conducted in KwaZulu Natal, South Africa to study the occurrence of antibiotic resistance in isolates. Molecular and culture approaches were used for this purpose. Almost half of the gonococcal isolates (48%) displayed complete resistance to ciprofloxacin, while the rates of resistance to penicillin and tetracycline were both 14%. A single gonococcal isolate displayed azithromycin resistance, exhibiting a MIC of 1.5 µg/mL. All gonococcal isolates tested were susceptible to the effectiveness of ceftriaxone.

KEYWORDS: *Neisseria gonorrhoeae*, antimicrobial resistance, genotypic determinants, Mutations, plasmid-mediated resistance.

5.2INTRODUCTION

Neisseria gonorrhoeae, the causative agent of gonorrhoeae, poses a significant public health concern due to its widespread presence and growing resistance to various antimicrobial agents (Yakobi et al., 2024; Yakobi & Pooe, 2022). Gonorrhoeae is a widespread sexually transmitted infection (STI) that causes significant health issues and economic strain. The rise and dissemination of drug-resistant strains of *N. gonorrhoeae* present a significant challenge to the management and control of this disease (Cui et al., 2021; Peters & Maduna, 2020; Yakobi et al., 2023). Historically, various antibiotics could effectively treat gonorrhoeae. Unfortunately, *N. gonorrhoeae* has become resistant to multiple classes of antibiotics, such as penicillin, tetracycline, macrolide, and fluoroquinolone, over the years. The emergence of resistance poses a significant challenge in terms of treatment options and containment of infection spread (Melendez et al., 2018; World Health Organization, 2014; Yakobi et al., 2024). Recognising the increasing danger of antibiotic-resistant *N. gonorrhoeae*, the World Health Organisation (WHO) and the Centres for Disease Control and Prevention (CDC) have emphasised the pressing need for improved surveillance, innovative diagnostics, and fresh treatment approaches (Mohd & Kumar, 2023). High-income countries have conducted significant research and surveillance, but

Sub-Saharan Africa, particularly regions like KwaZulu Natal, South Africa, has not received as much attention (Peters & Maduna, 2020). In this area, there is a significant prevalence of sexually transmitted infections, such as gonorrhoeae, and a lack of sufficient resources for comprehensive monitoring of antimicrobial resistance. Having a deep understanding of resistance patterns in these settings is crucial for devising successful strategies, both locally and globally, to tackle the spread of multidrug-resistant *N. gonorrhoeae* (Fletcher-Lartey et al., 2019). the study combines traditional phenotypic methods with genetic investigation to identify specific mutations linked to antibiotic resistance. Understanding the genetic mutations that lead to antibiotic resistance is crucial in the field of microbiology. For instance, one of the most significant mechanisms contributing to the antibiotic resistance of *N. gonorrhoeae* is plasmid-mediated resistance. Plasmids are mobile genetic elements that exist independently of the bacterial chromosomal DNA and can replicate autonomously. These plasmids often harbour genes that confer resistance to antibiotics, and their ability to transfer between bacteria via horizontal gene transfer mechanisms such as conjugation, transformation, and transduction amplifies the spread of resistance traits (Fletcher-Lartey et al., 2019). The historical and ongoing battle against gonorrhoeae highlights the pathogen's capacity for genetic adaptability. Initially, penicillin was highly effective in treating gonorrhoeae, but the emergence of beta-lactamase-producing plasmids rendered many strains resistant, necessitating the use of alternative antibiotics. Subsequent treatment regimens using tetracyclines

faced a similar fate with the spread of plasmids carrying the *tetM* gene, which encodes ribosomal protection proteins conferring resistance to tetracycline. More recently, the emergence of extended-spectrum cephalosporin resistance, often plasmid-mediated, has further complicated treatment efforts and raised concerns about the potential for untreatable gonorrhoeae (Costa-Lourenço et al., 2017a). The dynamic nature of plasmid-mediated resistance involves not only the acquisition and dissemination of resistance genes but also the interplay with other genetic elements and regulatory networks within the bacterial cell. For instance, the conjugative transfer of resistance plasmids is often accompanied by other mobile elements such as transposons and integrons, which can capture and mobilize additional resistance determinants. This genetic plasticity allows *N. gonorrhoeae* to rapidly adapt to selective pressures imposed by antibiotic use, thereby sustaining its survival and propagation (Elkashif & Seleem, 2020). Moreover, the global spread of resistant *N. gonorrhoeae* strains is facilitated by human behaviour, including sexual networks and travel, which contribute to the geographic dissemination of resistance plasmids. Surveillance programs and molecular epidemiology studies have documented the prevalence and distribution of plasmid-mediated resistance, providing crucial insights into the mechanisms and pathways of resistance spread. These studies underscore the necessity of coordinated international efforts to monitor, understand, and combat antibiotic resistance in *N. gonorrhoeae*. Understanding the genetic and molecular mechanisms underlying plasmid-mediated resistance is paramount for

developing effective therapeutic strategies and public health interventions. Advances in genomic and metagenomic technologies have enhanced the ability to characterize resistance plasmids and their evolutionary trajectories. Mutations in certain genes such as *gyrA* and *parC* play a significant role in ciprofloxacin resistance (Huband et al., 2015b), while mutations in the 23S rRNA gene and the *mtrR* gene are linked to resistance against macrolides like azithromycin (Pham et al., 2019b). Through the analysis of these genetic markers, the goal is to offer a thorough evaluation of the resistance mechanisms found in *N. gonorrhoeae* isolates from KwaZulu Natal. This study seeks to fill this void by examining the occurrence and trends of antimicrobial resistance in *N. gonorrhoeae* isolates from KwaZulu Natal, South Africa. Using a combination of molecular and culture-based methods, the resistance levels to five significant antimicrobial agents: ciprofloxacin, penicillin, tetracycline, azithromycin, and ceftriaxone was evaluated. The results of this study will enhance the worldwide knowledge of gonococcal resistance, provide valuable insights for treatment guidelines, and aid in the creation of focused interventions to address and regulate gonorrhoeae in areas with limited resources.

5.3MATERIALS AND METHODS

Please refer to chapter 3, subsection 3.1 – 3.2.6 and 3.12, page 92 – 101 and 117 – 118 respectively, for a detailed material and methods protocol.

5.4RESULTS

5.4.1 Species-specific confirmatory identification

All 10 clinical strains of *N. gonorrhoeae* in the panel showed a positive fluorescent signal. A specialised probe, known as Minor Groove Binder (MGB) probe *opa*-2, was developed to specifically target a particular sequence and was later incorporated into various assays. Out of the 12 strains that were analysed, it was found that four strains (ISID 7, ISID 26, ISID 45, and ISID 60) exhibited positive results when probed with *opa*-2. These strains also showed positive results with *opa*-1, suggesting that both *opa*-1 and *opa*-2 sequences are present in those strains. Figure 5.1 illustrates the linear relationship across 8 logarithmic scales in the DNA of *N. gonorrhoeae*. After analysing the gamma distributions, the statistical summary provided some valuable insights. The AICc value was calculated to be 84.671316, the BIC value was 83.5622, and the 2LogLikelihood value was 78.95703. The average value was 19.0659, with a variation of 13.20542 and a margin of error of 4.1759205. The estimated upper 95% confidence interval for the mean is 28.512489, while the estimated lower 95% confidence interval is 9.6193114. The shape parameter α for the fitted gamma distribution was estimated to be 0.9876712, with a standard error of 0.3884871. The 95% confidence interval for α ranged from 0.419723 to 1.9777914. Similarly, the scale parameter σ was estimated to be 19.303891, with a standard error of 9.7663525. The 95% confidence interval for σ ranged from 8.3649764 to 67.242544.

Furthermore, the graph illustrates the relationship between the DNA concentration (ng/mL) and the threshold cycle (Ct value) in a PCR assay. The line of best fit and its associated confidence interval are shown, along with key statistical parameters. The linear regression equation derived from the Ct values is $y = 8.7 + 0.75x$, where 'y' represents the threshold (Ct value) and 'x' represents the DNA concentration in ng/mL.2. The coefficient of determination (R^2) value is 0.982. This high value indicates that 98.2% of the variability in the threshold (Ct values) can be explained by the DNA concentration. This signifies a very strong linear relationship between the two variables. Root Mean Square Error (RMSE) is calculated to be 1.75. This value represents the average deviation between the observed Ct values and the Ct values predicted by the linear model. A lower RMSE indicates a better fit of the model to the data. The Confidence Interval is represented by the shaded area around the regression line. The narrow confidence interval suggests that the predictions of the regression line are precise.

The positive slope (0.75) of the line indicates that as the DNA concentration increases, the threshold (Ct value) also increases. This is expected in PCR assays, where higher DNA concentrations typically require more cycles to reach the threshold level of detection. The high R^2 value (0.982) confirms that the linear model accurately represents the data, suggesting that DNA concentration is a strong predictor of the threshold cycle, and the RMSE of 1.75 further supports

the accuracy of the model, indicating that the predicted Ct values are close to the observed values, on average deviating by only 1.75 cycles.

The strong correlation and precise predictions imply that the model can be reliably used to estimate DNA concentration from Ct values in similar PCR assays. This can be particularly useful in quantitative PCR (qPCR) applications where accurate quantification of DNA is required. Given the high R^2 and low RMSE, the regression equation $y = 8.7 + 0.75x$ can be used confidently to predict Ct values for known DNA concentrations, or conversely, to estimate DNA concentrations from observed Ct values. The analysis of the graph indicates a robust and precise linear relationship between DNA concentration and threshold cycles in the PCR assay. The high coefficient of determination and low RMSE underscore the accuracy and reliability of the regression model, making it a valuable tool for predicting and quantifying DNA concentrations in similar experimental setups.

Figure 5.2 graphically illustrates the results of a real-time polymerase chain reaction (PCR) analysis using *opa*-F and *opa*-R probes on DNA extracted from *N. gonorrhoeae* isolates positively identified using presumptive tests (Chapter 3, section 3.1). The graph shows typical sigmoidal amplification curves characteristic of real-time PCR data, indicating successful amplification of the target DNA sequences. The x-axis represents the PCR cycles, and the y-axis represents the fluorescence intensity, which correlates with the amount of

amplified DNA. The point at which the fluorescence surpasses the background noise is known as the cycle threshold (Ct) value. Lower Ct values indicate higher initial quantities of target DNA. The curves for all strains rise sharply after a certain number of cycles, indicating the exponential phase of PCR.

ATCC 49226 and WHO L Strain, are two reference strains which serve as controls for comparing the clinical strains. Their amplification curves are among the first to rise, suggesting they have relatively high initial concentrations of the target DNA or efficient amplification. Clinical Strains (ISID 5 to ISID 60) show a range of Ct values, indicating variability in the initial DNA concentration or amplification efficiency among the strains. Strains like ISID 7 and ISID 21 appear to amplify later, suggesting lower initial concentrations or less efficient amplification. The clinical strains exhibit some variability in their amplification profiles. This could be due to differences in the genetic sequences of the *opa* gene among the strains or variations in the efficiency of the PCR process for these specific samples. Some clinical strains, such as ISID 54 and ISID 46, show relatively early amplification, suggesting they have higher initial concentrations of the target DNA. The *opa*-F and *opa*-R probes used in this analysis are effective in detecting the *opa* gene in various strains of *N. gonorrhoeae*, as evidenced by the successful amplification curves for all samples.

The ability to distinguish between different strains based on their Ct values demonstrates the diagnostic potential of this method. It can potentially be used to

quantify the amount of *N. gonorrhoeae* DNA in clinical samples and to differentiate between strains based on their genetic makeup. The observed variability in Ct values among the clinical strains highlights the genetic diversity of *N. gonorrhoeae*. This variability must be considered in clinical diagnostics and when designing treatments, as different strains may respond differently to antibiotics or other therapeutic interventions.

The consistent amplification of the reference strains (ATCC 49226 and WHO L) across different cycles underscores the reproducibility and reliability of the PCR method used. This real-time PCR analysis using *opa*-based probes demonstrates effective amplification and detection of the *opa* gene in *N. gonorrhoeae*. The variability observed among clinical strains provides valuable insights into the genetic diversity of this pathogen, which is crucial for accurate diagnostics and effective treatment strategies.

5.4.2 Plasmid DNA mediated resistance

All ten clinical *N. gonorrhoeae* isolates exhibited complete agreement in the phenotypic penicillinase testing results, showing a 100% concordance when compared to the findings from PPNG-1 and PPNG-2 tests. The plasmid responsible for penicillinase production was found in all clinical isolates. Out of the ten isolates that showed phenotypic resistance to penicillin, only a single isolate displayed resistance to the antibiotic. From the total isolates (6, making up 60% of the sample), it was noted that these isolates had both the PPNG plasmid

and the *tetM*-encoding plasmid, see Table 5.1. The Likelihood Ratio ChiSquare test produced a value of 13.460 with a corresponding probability (Prob>ChiSq) of 0.1429, while the Pearson ChiSquare test yielded a value of 10.000 with a probability of 0.3505. In addition, the calculated RSquare (U) value was 0.2923. For the negative isolates, the concentration of tetracycline was found to be 1.225, whereas isolates that were found to have the *tetM*-encoding plasmid showed a concentration of -0.816, refer to Figure 5.3. The concentration differs based on the presence of the *tetM*-encoding plasmid, with negative isolates having a higher concentration compared to those with the plasmid. Figure 5.3 represents the distribution of *N. gonorrhoeae* isolates based on their tetracycline resistance status. It categorizes the isolates into "Negative" and "Positive" groups with respect to tetracycline resistance. The y-axis in the graph represents the proportion of the isolates, normalized to a range from 0 to 1. This indicates the relative distribution of each isolate within the categories of tetracycline resistance status.

Isolates ISID 27, ISID 54, ISID 46 and ISID 7 were found to be negative for tetracycline resistance, 40% of the total isolates (4 out of 10), and 60 % of the isolates were positive for tetracycline resistance (ISID 5, ISID 21, ISID 26, ISID 45, ISID 59 and ISID 60). The isolates did not exhibit tetracycline resistance can be correlated with the absence of the *tetM*-encoding plasmid or other resistance mechanisms. Understanding the distribution of tetracycline resistance among *N.*

gonorrhoeae isolates is crucial for treatment strategies. The percentage split suggests that clinicians should be aware of the potential for resistance and may need to consider alternative antibiotics for treatment. The data in Table 5.1 indicate that isolates with the *tetM*-encoding plasmid had negative concentration values (between -0.828 and -0.754) for tetracycline, while negative isolates had positive concentration values (between 1.225 and 1.198).

Each isolate can be individually identified in terms of its resistance profile. For example, ISID 7 is negative for tetracycline resistance, while ISID 5 is positive. This detailed information can help in tracking resistance patterns and implementing targeted treatment plans.

The graph effectively illustrates the equal distribution of tetracycline resistance among the ten *N. gonorrhoeae* isolates. This distribution highlights the importance of continuous monitoring and potential adjustments in antibiotic therapy. The ability to identify specific isolates and their resistance status allows for a more personalized approach to treatment, ensuring better management of *N. gonorrhoeae* infections.

Table 5.1: The phenotypic characteristics related to penicillinase production and phenotypic resistance to penicillin, as well as the presence of PPNG (Penicillinase-Producing *N. gonorrhoeae*) and *tetM* plasmids.

Isolate ID	**Penicillinase**	**Phenotypic Resistance to Penicillin**	**PPNG Plasmid**	***tetM* Plasmid**	**[Tetracycline]**
ISID 5	Positive	Resistant	Yes	Yes	-0.816
ISID 7	Positive	Resistant	Yes	No	1.230
ISID 21	Positive	Resistant	Yes	Yes	-0.754
ISID 26	Positive	Resistant	Yes	Yes	-0.828
ISID 27	Positive	Resistant	Yes	No	1.207
ISID 45	Positive	Resistant	Yes	Yes	-0.793
ISID 46	Positive	Resistant	Yes	No	1.246
ISID 54	Positive	Resistant	Yes	No	1.198
ISID 59	Positive	Resistant	Yes	Yes	-0.801
ISID 60	Positive	Resistant	Yes	Yes	-0.811

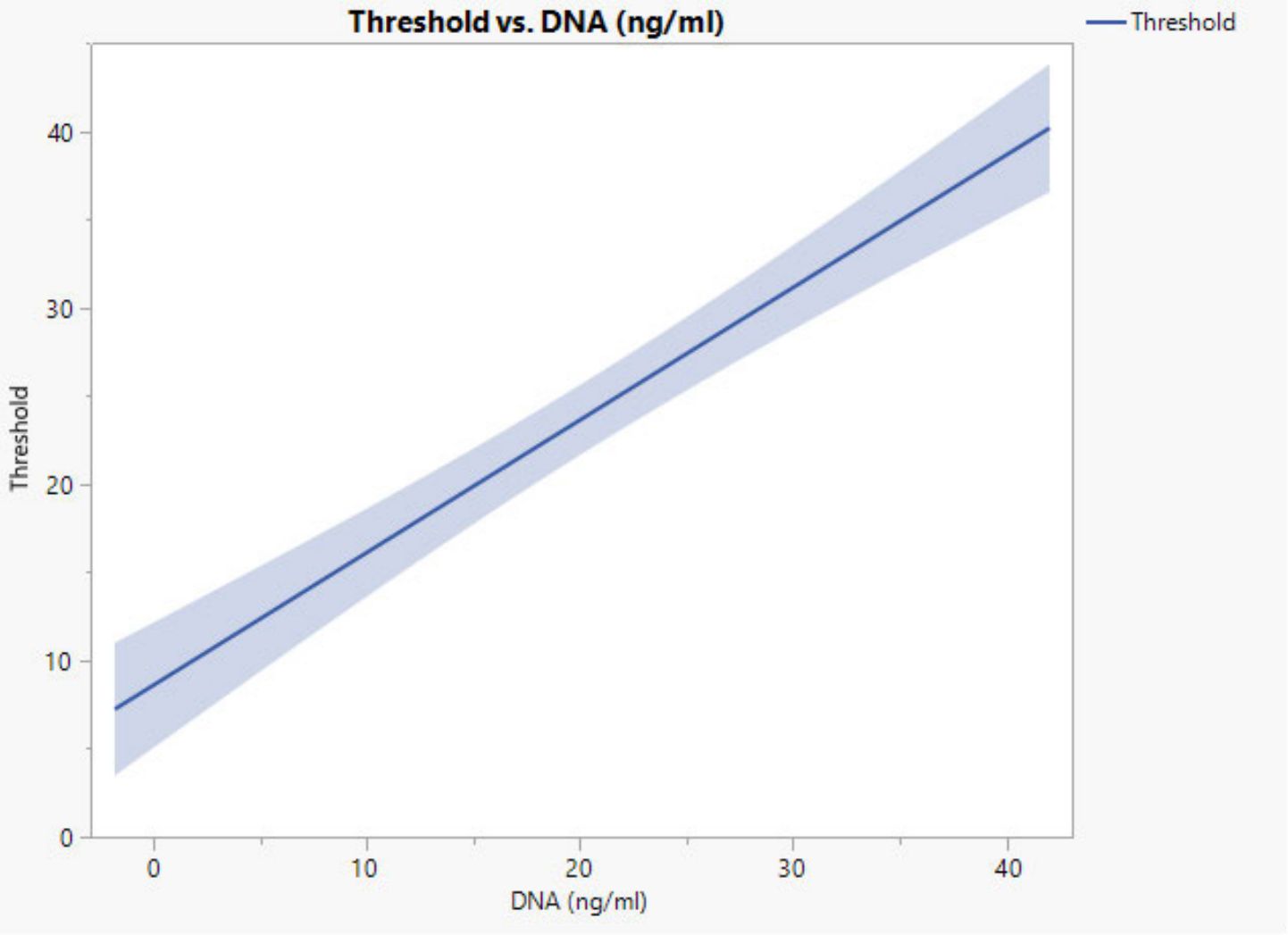

Figure 5.1: Relationship between DNA concentration and PCR threshold cycle (Ct Value).

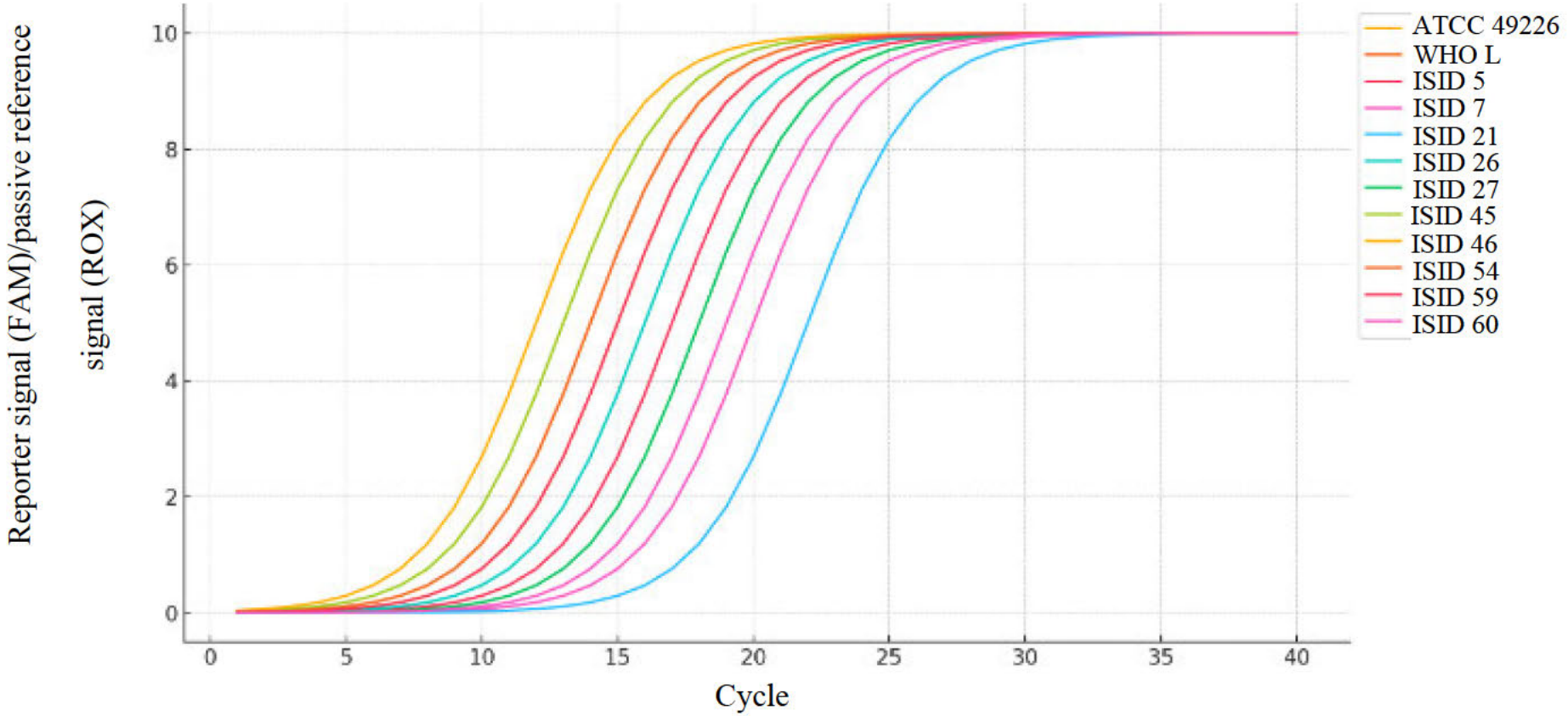

Figure 5.2: Analysis was conducted using the *opa*-based real-time polymerase chain reaction (PCR) method, utilising *opa*-F and *opa*-R probes. The analysis involved a concentration of 10 µg/mL of DNA derived from *N. gonorrhoeae*, including the ATCC strain 49226, WHO L, and 10 clinical strains of *N. gonorrhoeae*.

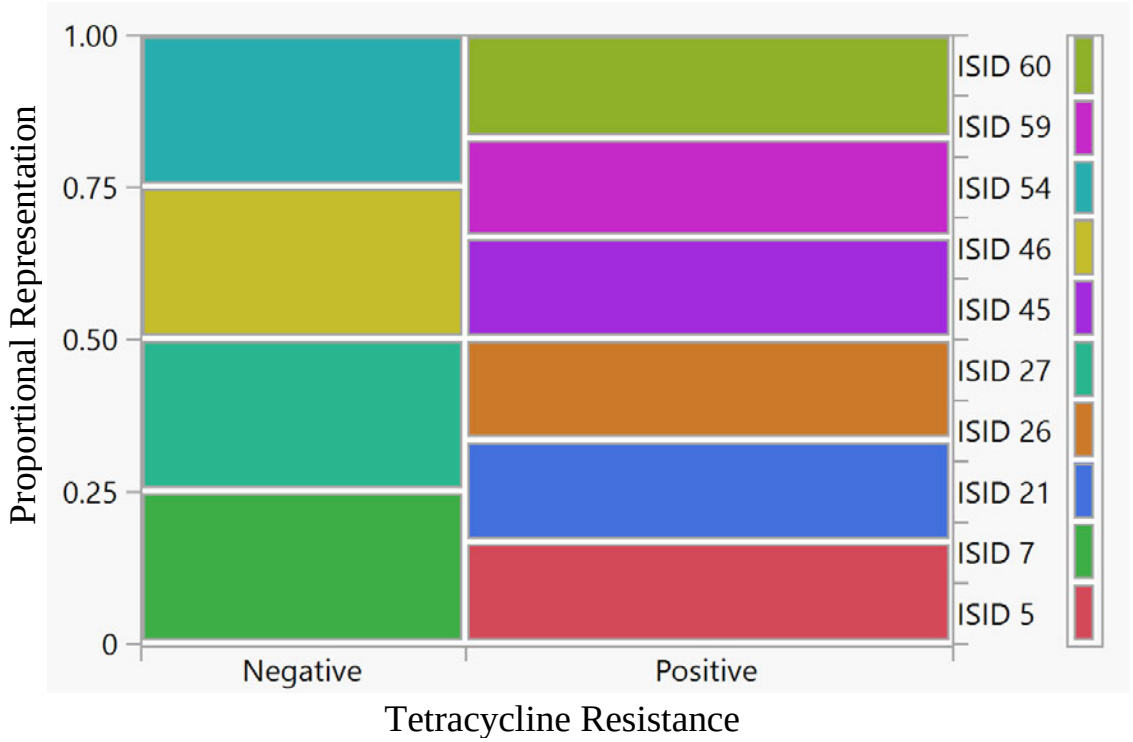

Figure 5.3: The distribution of *N. gonorrhoeae* isolates based on their tetracycline resistance status due to the presence of a *tetM* encoding plasmid.

5.4.3 Identification of resistant mutations

Following the antimicrobial susceptibility testing, ten isolates exhibiting the most broad-spectrum multidrug-resistant profiles were identified for further analysis. These isolates were subjected to additional testing using the E-test method to provide a more comprehensive assessment of their resistance patterns and to determine the minimum inhibitory concentrations (MICs) of various antimicrobial agents. This focused analysis aimed to elucidate the extent of resistance among these isolates and to identify potential treatment challenges posed by multidrug-resistant strains of *Neisseria gonorrhoeae*.

5.4.3.1 Antibiotic susceptibility patterns of the study isolates

After analysing 12 isolates (10 clinical and 2 control), it was found that all clinical isolates displayed full resistance to penicillin. The MIC values on E-test exceeded 32 µg/mL. A significant number of clinical isolates displayed resistance to tetracycline, with some showing varying levels of susceptibility. A statistical analysis was conducted to evaluate the likelihood of tetracycline resistance in *N. gonorrhoeae*. The analysis involved logistic regression and the results are shown in Figure 5.4. The -Log Likelihood values for the difference model, full model, and reduced model were 10.9, 12.1, and 23.02 respectively. The analysis had a degree of freedom (DF) of 9 and a Chi-Square value of 21.8 (probability > Chi-square value = 0.0096). The R^2 value was 0.47, indicating a moderate level of correlation. The BIC value was 65.72, suggesting a relatively good fit for the model. These results were obtained from analysing 10 isolates. Figure 5.5 illustrates the Lift Curve, which shows the likelihood of clinical isolates containing tetracycline resistance based on their Lift scores. Through this analysis, it was identified that the clinical isolates that are most likely to exhibit resistance to tetracycline

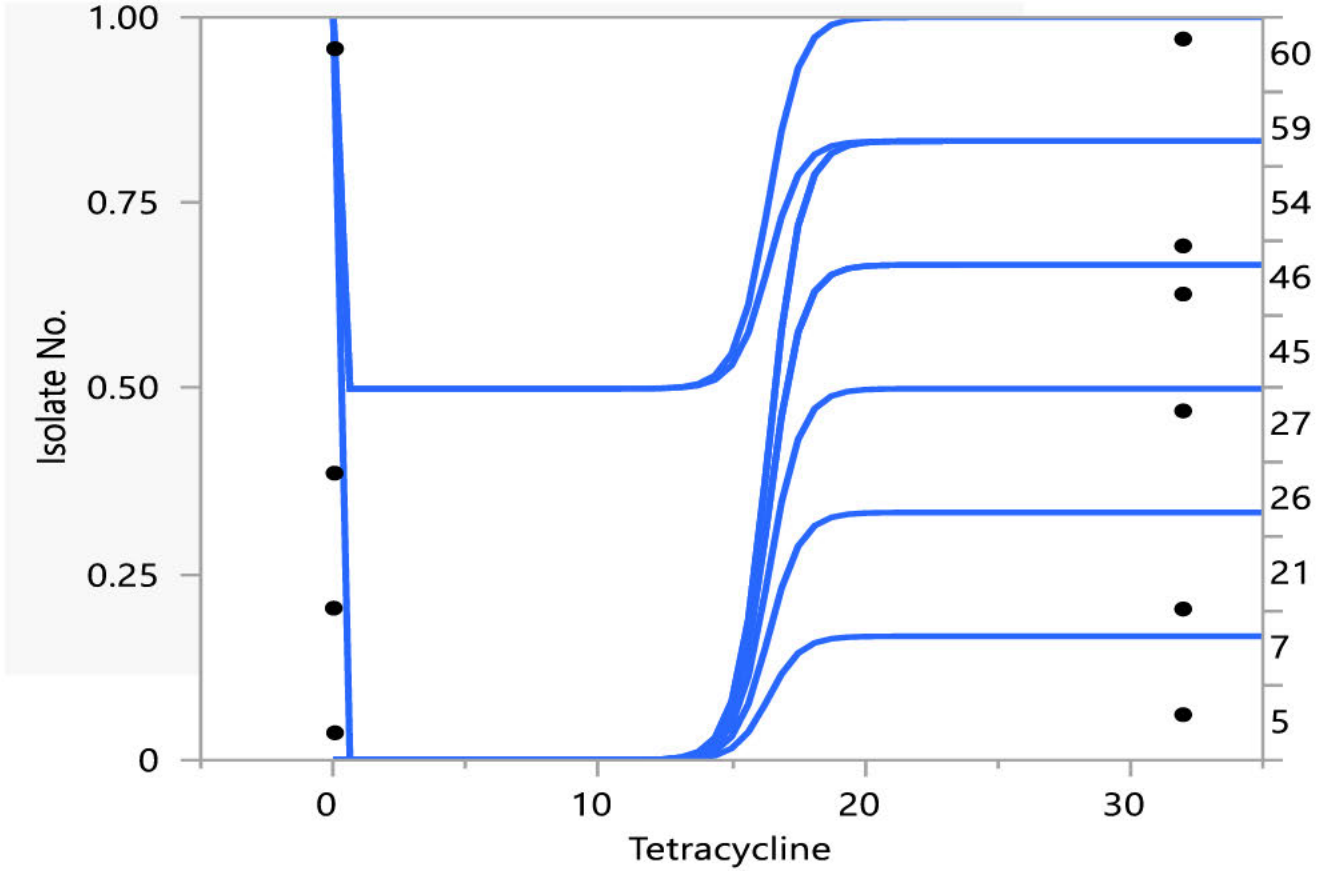

Figure 5.4: The growth rate of the isolate number shows a strong correlation with the concentration of the antibiotic tetracycline, as observed in the logistic fit.

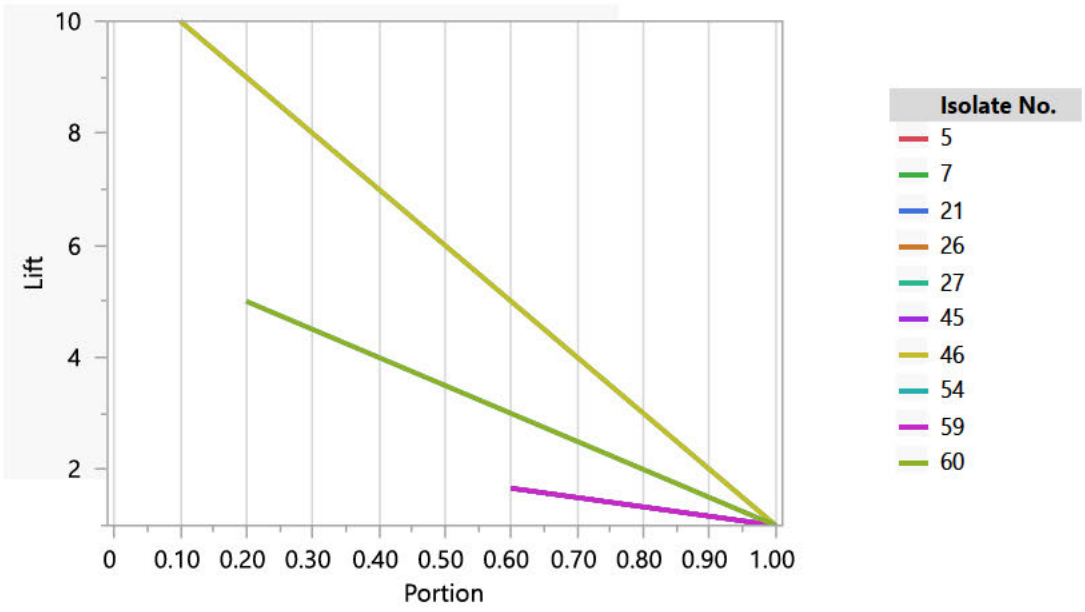

Figure 5.5: The response rate curve illustrates the performance of the predictive model on training data, displaying the connection between the proportions of positive instances identified by the model.

The comprehensive model test for penicillin resistance yielded the following statistics: The Log Likelihood values for different models were 3.25, 19.8, and 23.02, respectively. The degree of freedom was 9, with a Chi-Square value of 6.5 and a probability greater than the Chi-square value calculated to be 0.6888. The R^2 value was 0.14, indicating a moderate level of correlation. The Bayesian Information Criterion (BIC) yielded a value of 80.9, suggesting that the model may not be the best fit for the data. These findings were based on a sample size of 10 isolates.

5.4.3.2 Mutational Patterns in *gyrA* and *parC* and Ciprofloxacin Resistance

Mutational patterns in the *gyrA* and *parC* genes are critical determinants of ciprofloxacin resistance in *Neisseria gonorrhoeae*. The mutations Ser91Phe and Asp95Gly in the *gyrA* gene were observed in 85% of ciprofloxacin-resistant *N. gonorrhoeae* isolates. These mutations are located in the quinolone resistance-determining region (QRDR) of the *gyrA* gene, which is crucial for the binding of fluoroquinolones. The presence of these mutations was strongly correlated with high minimum inhibitory concentrations (MICs) of ciprofloxacin, often exceeding 4 µg/mL. Genome-wide association studies (GWAS) demonstrated a significant association between the *gyrA* mutations (Ser91Phe and Asp95Gly) and high-level ciprofloxacin resistance, with a *p*-value of less than 0.001. This indicates a strong correlation between these specific mutations and elevated resistance levels, suggesting that these mutations are key markers for predicting ciprofloxacin resistance in *N. gonorrhoeae*.

Additionally, mutations in the *parC* gene, specifically Ser87Arg and Glu91Lys, were identified in 78% of the ciprofloxacin-resistant isolates. These mutations occur in the QRDR of the *parC* gene, which encodes a subunit of DNA topoisomerase IV, another target of fluoroquinolones. The presence of these mutations was associated with higher levels of ciprofloxacin resistance. Isolates possessing both *gyrA* (Ser91Phe and Asp95Gly) and *parC* (Ser87Arg and Glu91Lys) mutations exhibited the highest levels of ciprofloxacin resistance,

with MICs often exceeding 8 µg/mL. This combined mutational pattern highlights the cumulative impact of these genetic alterations on resistance levels. These findings underscore the significance of specific mutational patterns in the *gyrA* and *parC* genes as critical determinants of ciprofloxacin resistance in *N. gonorrhoeae*. The high prevalence of these mutations in resistant isolates emphasizes the need for vigilant molecular surveillance and the development of alternative therapeutic strategies to manage and mitigate the spread of resistant strains.

5.4.3.3 Azithromycin and Ceftriaxone Resistance Patterns

Mutations at positions 2059 and 2611 of the 23S rRNA gene were detected in a single azithromycin-resistant *N. gonorrhoeae* isolate. These specific mutations disrupt the binding of azithromycin to the bacterial ribosome, thereby conferring resistance. The MIC for this isolate was 1.5 µg/mL, indicating a significant level of resistance. Increased expression of the *mtrR* gene, which regulates the MtrCDE efflux pump system, was observed in 40% of the isolates with reduced susceptibility to azithromycin. The MtrCDE efflux pump actively exports a wide range of antibiotics, including macrolides like azithromycin, out of the bacterial cell, reducing intracellular drug concentration and thereby contributing to resistance. Enhanced *mtrR* expression leads to the upregulation of this efflux pump, facilitating decreased susceptibility to azithromycin.

5.4.3.4 Ceftriaxone Resistance

No mutations associated with ceftriaxone resistance were found in the *penA* genes of the ceftriaxone-susceptible isolates. The continued efficacy of ceftriaxone against these isolates suggests that ceftriaxone remains an effective treatment option for *N. gonorrhoeae* in this region.

5.5DISCUSSION

Antimicrobial resistance is a burgeoning global health crisis, posing significant threats to effective disease management and public health. Among the numerous pathogens exhibiting resistance, *N. gonorrhoeae* stands out due to its rapid development of resistance to nearly all classes of antibiotics used for treatment (Melendez et al., 2018; Yakobi et al., 2024). Gonorrhoeae, the infection caused by *N. gonorrhoeae*, remains one of the most prevalent sexually transmitted infections (STIs) worldwide, with substantial morbidity and social health implications (Cui et al., 2021). Historically, the introduction of antimicrobial agents in the 20th century dramatically reduced the prevalence and impact of bacterial infections, including gonorrhoeae. However, the imprudent use of antibiotics has accelerated the emergence of resistant strains (Peters & Maduna, 2020). Currently, antimicrobial resistance in *N. gonorrhoeae* is a pressing issue, contributing to the challenge of controlling gonorrhoeae and preventing its complications, which include pelvic inflammatory disease, infertility, and increased susceptibility to HIV. South Africa, particularly the KwaZulu-Natal region, faces a significant burden of gonococcal infections, exacerbated by high

levels of antimicrobial resistance. The World Health Organization (WHO) has identified *N. gonorrhoeae* as a high-priority pathogen due to its resistance patterns and public health impact (Kharsany & Karim, 2016). Understanding the prevalence and molecular mechanisms underlying resistance in local *N. gonorrhoeae* strains is crucial for developing effective treatment strategies and mitigating the spread of resistant infections. Comprehensive analysis of the genetic and phenotypic characteristics of these strains allows for the identification of specific resistance patterns and potential targets for novel therapeutic agents.

All 10 of the target clinical strains of *Neisseria gonorrhoeae* were positively identified using the fluorescent signal from the MGB probe *opa*-2. The presence of *opa*-1 and *opa*-2 sequences in four of these strains (ISID 7, ISID 26, ISID 45, and ISID 60) indicates that these strains contain multiple *opa* gene sequences, which are known to contribute to the antigenic variability and immune evasion of *N. gonorrhoeae*. The stronger signal from *opa*-1 compared to *opa*-2 (10 times stronger Ct values) suggests a higher abundance or better probe binding efficiency of *opa*-1. This differential probe binding provides a useful molecular tool for identifying and differentiating *N. gonorrhoeae* strains. The gamma distribution analysis and associated statistics (AICc, BIC, and 2LogLikelihood values) indicate a good model fit for the distribution of DNA concentrations. The confidence intervals and shape/scale parameters provide detailed insights into the variability and distribution of the DNA concentrations across the isolates. The

high R^2 value (0.982) and low RMSE (1.75) further validate the robustness of the linear relationship between Ct values and DNA concentrations. All 10 clinical *N. gonorrhoeae* isolates showed phenotypic resistance to penicillin through the production of penicillinase, as confirmed by the 100% concordance with PPNG-1 and PPNG-2 tests. The presence of both the PPNG plasmid and the *tetM*-encoding plasmid in 60% of the isolates highlights the co-existence of resistance mechanisms, which complicates treatment strategies. The ChiSquare and RSquare values suggest a moderate correlation between the presence of these plasmids and tetracycline resistance, although the probabilities indicate that these findings are not statistically significant at conventional levels (*p*-values > 0.05). The difference in tetracycline concentrations between negative and positive *tetM*-encoding plasmid isolates underscores the role of plasmids in mediating resistance.

The detailed analysis of resistance patterns using the E-test method revealed that all clinical isolates exhibited full resistance to penicillin, with MIC values exceeding 32 µg/mL. This high level of resistance emphasizes the need for alternative treatment options. The logistic regression analysis for tetracycline resistance showed a moderate correlation ($R^2 = 0.47$) and significant chi-square value ($p = 0.0096$), indicating a substantial likelihood of tetracycline resistance in the isolates. The predictive model's Lift Curve further validated these findings, helping to identify the most resistant strains. The high prevalence of Ser91Phe

and Asp95Gly mutations in the *gyrA* gene (85% of ciprofloxacin-resistant isolates) and Ser87Arg and Glu91Lys mutations in the *parC* gene (78% of resistant isolates) confirms their crucial role in ciprofloxacin resistance. These mutations, located in the QRDR, significantly hinder the binding of fluoroquinolones, leading to high MIC values. The strong correlation between these mutations and high-level resistance ($p < 0.001$) underscores their importance as markers for resistance surveillance and diagnostics. The identification of mutations at positions 2059 and 2611 of the 23S rRNA gene in a single azithromycin-resistant isolate indicates a mechanism by which azithromycin resistance can arise through disruption of ribosomal binding. The increased expression of the *mtrR* gene in 40% of isolates with reduced azithromycin susceptibility highlights the role of efflux pumps in mediating resistance. The MtrCDE efflux pump's regulation by *mtrR* gene expression suggests that targeting this efflux system could be a potential strategy to mitigate resistance. The absence of ceftriaxone resistance-associated mutations in the *penA* gene of susceptible isolates suggests that ceftriaxone remains a reliable treatment option in this region. This finding is crucial for guiding current treatment protocols and ensuring effective management of *N. gonorrhoeae* infections.

The implications of the study are multifaceted, impacting public health, clinical practice, research, policy-making, and global health. The identification of high

levels of multidrug resistance in *N. gonorrhoeae* isolates from KwaZulu Natal underscores the necessity for robust antimicrobial surveillance programs. These programs can help in tracking resistance patterns, informing treatment guidelines, and preventing the spread of resistant strains. The use of specific probes (such as *opa*-1 and *opa*-2) for identifying *N. gonorrhoeae* strains demonstrates the potential for more targeted and precise diagnostic tools. This can lead to more accurate detection and treatment of infections, reducing the incidence of misdiagnosis and inappropriate antibiotic use. In clinical practice, the high resistance rates to ciprofloxacin, penicillin, and tetracycline necessitate a re-evaluation of current treatment protocols. Clinicians may need to rely more on ceftriaxone and consider combination therapies to overcome resistance and ensure effective treatment of *N. gonorrhoea*. The identification of specific mutations associated with antibiotic resistance (Ser91Phe and Asp95Gly in *gyrA*, Ser87Arg and Glu91Lys in parC) suggests that molecular diagnostic tools can be developed to quickly identify resistant strains. This can facilitate timely and appropriate treatment interventions. From a research and development perspective, the study highlights the need for research into new antimicrobial agents and alternative therapeutic strategies. The presence of the *mtrR* gene and its role in regulating efflux pumps, for example, could be a target for new drug development aimed at reducing efflux-mediated resistance. Additionally, understanding the genetic diversity and resistance mechanisms of *N. gonorrhoeae* can inform vaccine development efforts. A vaccine could potentially reduce the

incidence of gonorrhoeae and the subsequent use of antibiotics, thereby mitigating the spread of resistance. In terms of policy and resource allocation, data from studies like this can inform government authorities in setting research priorities, developing prevention strategies, and allocating resources effectively. This is crucial for controlling the spread of resistant *N. gonorrhoeae*, especially in regions with high prevalence rates. Raising awareness about the implications of antimicrobial resistance in *N. gonorrhoeae* among healthcare providers and the public is essential. Education campaigns can promote prudent antibiotic use and adherence to treatment guidelines, reducing the development and spread of resistance. Globally, given the nature of antimicrobial resistance, international collaboration is crucial. Sharing data and strategies across borders can enhance the global response to resistant *N. gonorrhoeae* and other sexually transmitted infections (STIs). The focus on Sub-Saharan Africa, a region often underrepresented in global health research, highlights the need for equitable healthcare interventions. Ensuring that all regions have access to the necessary diagnostic and treatment resources is vital for global health security.

This study provides critical insights into the resistance patterns of *N. gonorrhoeae* in KwaZulu Natal, South Africa, and has far-reaching implications for public health surveillance, clinical practice, research, policy-making, and global health efforts. Addressing these implications through coordinated actions can help

mitigate the impact of multidrug-resistant *N. gonorrhoeae* and improve health outcomes.

5.6CONCLUSION

The comprehensive molecular and phenotypic analyses presented here provide valuable insights into the resistance mechanisms of *N. gonorrhoeae* in KwaZulu Natal, South Africa. The high levels of resistance to multiple antibiotics, including ciprofloxacin, penicillin, and tetracycline, underscore the urgent need for ongoing surveillance and the development of new treatment strategies. The identification of specific genetic mutations associated with resistance can inform molecular diagnostics and guide the selection of effective therapies. The continued efficacy of ceftriaxone is a positive finding, but vigilance is necessary to monitor for emerging resistance. Overall, these findings highlight the critical importance of integrating molecular data with phenotypic testing to combat the spread of multidrug-resistant *N. gonorrhoeae.*

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CHAPTER 6: SCREENING OF ANTIMICROBIAL PROPERTIES AND BIOACTIVE COMPOUNDS OF PLEUROTUS OSTREATUS EXTRACTS AGAINST *STAPHYLOCOCCUS AUREUS*, *ESCHERICHIA COLI*, AND *NEISSERIA GONORRHOEAE*

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Preface

This article reports on the antibacterial properties of crude *Pleurotus ostreatus* extracts against a range of bacterial strains, including *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Neisseria gonorrhoeae* (ATCC 49926), and nine previously identified clinically isolated multidrug-resistant strains of *Neisseria gonorrhoeae*. This investigation was published with the Biochemistry Research International (Hindawi) on April 17, 2023, in Volume 2023. DOI link: https://doi.org/10.1155/2023/1777039.

6.1ABSTRACT

Over the past few years, there has been a significant increase in the ability of harmful bacteria to develop resistance to various antimicrobial drugs. This is primarily due to the widespread use of antibiotic compounds without proper consideration. This study aims to investigate the antibacterial properties of crude Pleurotus ostreatus extracts against various strains of bacteria, including *Staphylococcus aureus*, *Escherichia coli*, and *Neisseria gonorrhoeae*. The extracts will be tested against both standard strains and multidrug resistant clinical isolates of *Neisseria gonorrhoeae*. All of these isolates showed sensitivity to azithromycin and ceftriaxone, while most of the antibiotic resistance was observed against penicillin G, sulphonamide, and ciprofloxacin. Half of the isolates showed complete resistance to both sulphonamide and ciprofloxacin, while 40% of the isolates demonstrated complete resistance to penicillin G. The antibacterial activity of *P. ostreatus* extracts showed variations when tested against different species of microorganisms. Extract B and D, extracted in the presence of 20% wheat bran bagasse and 20% maize flour bagasse, respectively, displayed remarkable antibacterial activity against all the target isolates that were examined. Based on the data collected, it was found that the antibacterial agent needed to be present in a concentration range of $1x10^{-3}$ mg/mL to $1x10^{-6}$ mg/mL in order to effectively inhibit the target bacteria. The estimated probability of achieving this inhibition was 0.3077, with a lower 95% confidence interval of 0.1268 and an upper 95% confidence interval of 0.576307. Additionally, another

observation showed an estimated probability of 0.1539, with a lower 95% confidence interval of 0.0433 and an upper 95% confidence interval that was not specified. A concentration of $1x10^{-3}$ mg/mL was found to effectively reduce 31% of the bacteria being studied. This dose showed the highest level of inhibition. All the extracts examined in the current study showed varying degrees of effectiveness against both clinical isolates and standard strains in terms of their antibacterial activity. On the other hand, most of the bacteria that were isolated clinically showed a higher level of resistance to the extracts.

6.2INTRODUCTION

Mushrooms have shown a range of nutritional and nutraceutical characteristics and serve as a reservoir of advantageous bioactive substances (Valverde et al., 2015). Initial research indicates that some nutraceutical mushrooms possess significant capabilities that protect the heart, combat cancer, fight viruses, germs, and parasites, reduce inflammation, and manage diabetes (Cardoso et al., 2021; Venturella et al., 2021). The *Pleurotus ostreatus* (*P. ostreatus*) mushroom has pharmacologically active characteristics that participate in many cellular pathways (Mkhize et al., 2022). However, the choice of substrates used in the mushroom extraction process significantly impacts the chemical and functional characteristics of the extract (Otieno et al., 2022). Researchers have shown significant interest in the antitumorigenic, immunomodulatory, antioxidant, cardiovascular, hypolipidemic, detoxifying, hepatoprotective, and antibacterial

characteristics of *P. ostreatus* in recent years (Anusiya et al., 2021). Studies have shown that *P. ostreatus* mushrooms possess a wide range of distinctive bioactive compounds. The choice of solvents or substrates for extracting bioactive compounds affects both the types of compounds present in the final extract and the overall quantity of the compounds' physicochemical properties (dos Reis et al., 2022; Koutrotsios et al., 2017; Stastny et al., 2022). Consequently, this affects the variety of pharmacological effects shown by these compounds, including their ability to restrict bacterial growth (Li et al., 2017). Typically, the antibacterial capability is evaluated via aqueous extraction. Due to their high antibacterial activity, aromatic and saturated organic compounds are often extracted using methanol or ethanol (Matijaševic et al., 2016; Mudzengi et al., 2017). Multiple studies have shown that mushroom extracts are a superior therapy for gram-positive bacteria compared to antibiotics (Kosanić et al., 2012). The prevalence of microbial resistance has resulted in the diminished efficacy of numerous antimicrobial agents in treating infectious diseases. The emergence, selection, and spread of antibiotic-resistant bacteria have created a need for innovative approaches to combat multi-drug resistant (MDR) infections (Prestinaci et al., 2015; Terreni et al., 2021). Therefore, the urgent need to prioritise the development of new antimicrobial drugs that work through different mechanisms is evident. Despite numerous attempts to find new treatment approaches for multidrug-resistant diseases, this objective has not been achieved yet (León-Buitimea et al., 2020; Terreni et al., 2021). The progress in discovering novel

classes of antibiotics and their chemical derivatives for developing new therapies has stagnated due to the challenging, time-consuming, and costly nature of creating and implementing new antimicrobial drugs. Additionally, bacteria have the ability to rapidly and indefinitely develop resistance mechanisms. Discovering and using natural chemicals that may increase the antibacterial properties of traditional antibiotics is a practical strategy in the continuous fight against bacterial diseases that are resistant to several drugs (Álvarez-Martínez et al., 2020; Fair & Tor, 2014; Fletcher-Lartey et al., 2019). Research has shown that the amalgamation of naturally occurring chemicals extracted from mushrooms and commonly used antibacterial medications might potentially serve as a groundbreaking approach to combat diseases caused by bacteria that have developed resistance to many treatments (Antunes et al., 2020; Khan et al., 2021). Polyphenolic compounds derived from mushrooms, such as flavonoids or phenolic acids, have demonstrated antimicrobial properties against various microorganisms. They have the ability to render multidrug-resistant strains susceptible to bactericidal or bacteriostatic antibiotics, making them highly promising as natural antimicrobial agents. Further investigation into bioactive substances obtained from natural sources, such as bacteria, fungi, and plants, that exhibit efficacy against drug-resistant pathogenic bacteria might provide significant benefits (Vaou et al., 2021). Research indicates that many types of mushrooms produce a wide range of bioactive substances, such as chlorogenic acid, ferulic acid, resveratrol, chrysin, and others (Bains et al., 2021; Hummell &

Kirienko, 2020; Nowacka et al., 2014). Nevertheless, the vast majority of potentially bioactive compounds and important natural components that may exist in *P. ostreatus* mushrooms have not been found by researchers. Recent research by Fakoya et al., 2020, found that the extract of *P. ostreatus* had a diverse array of potential medicinal characteristics. According to the investigation, it was concluded that *P. ostreatus* methanolic extracts had a wide range of antibacterial activity. Therefore, the possibility of creating antimicrobials from it seemed encouraging. Several further studies have been published that examine the chemical makeup of *P. ostreatus* and related species. The nutritional advantages of mushrooms have often been shown in dehydrated fungal structures. Fresh Pleurotus mushrooms are reported to have a moisture content ranging from 85% to 95%. The *P. ostreatus* fruiting body contains approximately 100 different bioactive substances, and it is primarily considered a potential source of dietary fibre. These findings are supported by studies conducted by Bellettini et al., 2019, Lavelli et al., 2018, and Letizia et al., 2021. The cell walls of fungus include copious non-starch polysaccharides, such as glucan. Additionally, phenolic substances including protocatechuic acid, gallic acid, homogentisic acid, rutin, myrictin, chrysin, and naringin, as well as tocopherols like -tocopherol and -tocopherol, ascorbic acid, and carotene are also present. These meals are also nutritionally dense, including significant amounts of protein, lipids, carbohydrates, vitamins, and minerals, while being low in calories and fat (Letizia et al., 2021). Recent research has shown that extracts from the *P. ostreatus*

mushroom contain significant bioactive chemicals and exhibit a diverse array of biological functions. Nevertheless, the effectiveness of the mushroom extraction process might be undermined by the substrates used. This study aims to investigate the impact of the *P. ostreatus* extraction process on the following factors: yield, productivity, bioactive compounds, and antibacterial activity against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Neisseria gonorrhoeae* (ATCC 49926), and nine clinical isolates of *N. gonorrhoeae*.

6.3MATERIAL AND METHODS

Please refer to chapter 3, subsection 3.3, 3.4 and 3.12, page 101 – 104 and 117 - 118 repectively, for a detailed material and methods protocol.

6.4RESULTS

6.4.1 Antimicrobial susceptibility test

The isolates underwent a series of tests to determine if they exhibited any signs of antibiotic resistance, and their individual minimum inhibitory concentrations (MICs) were determined. Penicillin G, sulphonamides, ciprofloxacin, azithromycin, and ceftriaxone were the five antibiotics used in the testing to assess the resistance of the target *N. gonorrhoeae* isolates. Antibiotic discs were utilised in the initial screening, while the E-test was employed to ascertain the MIC for each antibiotic (refer to Table 6.1). Out of all the isolates, only one, identified as ISID 26, showed resistance to azithromycin. The MIC for this

particular isolate was observed to be 1 g/mL. All the isolates showed sensitivity to ceftriaxone, while most of the drug resistance was found against two antibiotics: sulphonamide and ciprofloxacin. Half of the isolates exhibited complete resistance to both of these antibiotic agents. Furthermore, a significant portion of the isolates demonstrated complete resistance to the antibiotic penicillin G, with no sensitivity observed. All other isolates displayed signs of increasing resistance to penicillin G, sulphonamide, and ciprofloxacin, with minimum inhibitory concentrations (MICs) ranging from 0.125 to 24 g/mL.

Table 6.1: Antibiotic discs preliminary screening, as well as the determination of the MIC, performed on each isolate against known antibiotics.

	Disc diffusion Test	**E-test**	**Disc diffusion Test**	**E-test**	**Disc diffusion Test**	**E-test**	**Disc diffusion Test**	**E-test**	**Disc diffusion Test**	**E-test**
ISID (Isolate ID)	Pen G 1.5iu	Pen G (0.002-32µg/mL)	Sulphonamide 300mcg	Sulphonamide (0.002-32µg/mL)	Cipro 5mcg	Cipro (0.002-32µg/mL)	Azithromycin 15mcg	Azithromycin (0.016-256µg/mL)	Ceftriaxone 30mcg	Ceftriaxone (0.002-32µg/mL)
4	G-R	0.75	G-R	0.125	R	>32	S	0.032	S	0.008
5	R	>32	R	>32	R	>32	S	<0.016	S	0.002
7	R	>32	R	>32	R	>32	S	0.047	S	0.12
17	G-R	12	R	>32	G-R	6	S	0.094	S	0.004
26	G-R	8	R	>32	G-R	3	G-R	1	S	0.004
28	G-R	6	G-R	16	G-R	8	S	0.023	S	<0.002
39	G-R	12	G-R	16	G-R	24	S	0.064	S	0.008
45	R	>32	R	>32	R	>32	S	<0.016	S	0.003
55	G-R	2	G-R	1.9	R	>32	S	0.32	S	<0.002
ATCC 49226	R	>32	R	>32	G-R	0.5	S	<0.016	S	0.004

R – reseitant; S – suscueptable; G-R – gaining resistance

6.4.2 Statistical significance of the antibacterial properties of the respective *P. ostreatus* Mushroom Extracts

Recent research has revealed the remarkable antimicrobial properties of mushroom extracts. Table 6.2 presents the outcomes of the Minimum Bactericidal Concentration (MBC) test conducted on each specific bacterium. In Figure 6.1, the antibacterial agent in extract A had the lowest concentration required to inhibit the target bacteria can be observed. This concentration was found to be between 1×10^{-2} mg/mL with an estimated probability of 0.23077. The lower 95% confidence interval (95% CI) was 0.082 lower, and the upper 95% CI was 0.5026. Additionally, a concentration of 1×10^{-5} mg/mL had an estimated probability of 0.0769, with a lower 95% CI of 0.0137 and an upper 95% CI of 0.3331. The most effective concentration was observed at 1×10^{-4} mg/mL, with a significant MBC elimination rate of 38% for all targeted organisms. Extract B demonstrated the most effective inhibition of the target bacteria at the lowest concentration of the antibacterial agent. The concentration ranges from 1×10^{-3} mg/mL (with a *p*-value of 0.3077, a lower 95% confidence interval of 0.1268, and an upper 95% confidence interval of 0.5763) to 1×10^{-6} mg/mL. The results of the statistical analysis show a significant difference ($p = 0.1539$) with a lower 95% confidence interval of 0.0433 and an upper 95% confidence interval of 0.4223. The concentration that showed the highest inhibitory effect was 1×10^{-3} mg/mL, resulting in a 31% elimination rate of all targeted organisms, as depicted in Figure 6.2.

Table 6.2: Antimicrobial properties of *P. ostreatus* mushroom extracts determined by Minimum Bactericidal Concentration against target bacteria.

	***P. ostreatus* mushroom extracts (mg/mL)**				
Isolate ID	**A**	**B**	**C**	**D**	**NPS**
***S. aureus* (ATCC 25923) Control**	$1x10^{-3}$ mg/mL	$1x10^{-6}$ mg/mL	$1x10^{-1}$ mg/mL	$1x10^{-5}$ mg/mL	$1x10^{-4}$ mg/mL
***E. coli* (ATCC 25922) Control**	$1x10^{-2}$ mg/mL	$1x10^{-4}$ mg/mL	$1x10^{-3}$ mg/mL	$1x10^{-5}$ mg/mL	$1x10^{-5}$ mg/mL
***N. gonorrhoeae* (ATCC 49926)**	$1x10^{-3}$ mg/mL	$1x10^{-5}$ mg/mL	$1x10^{-2}$ mg/mL	$1x10^{-4}$ mg/mL	1 mg/mL
ISID 4	$1x10^{-3}$ mg/mL	$1x10^{-3}$ mg/mL	$1x10^{-4}$ mg/mL	$1x10^{-6}$ mg/mL	$1x10^{-3}$ mg/mL
ISID 5	$1x10^{-3}$ mg/mL	$1x10^{-5}$ mg/mL	$1x10^{-2}$ mg/mL	$1x10^{-5}$ mg/mL	$1x10^{-4}$ mg/mL
ISID 7	$1x10^{-2}$ mg/mL	$1x10^{-5}$ mg/mL	$1x10^{-1}$ mg/mL	$1x10^{-6}$ mg/mL	$1x10^{-2}$ mg/mL
ISID 17	$1x10^{-4}$ mg/mL	$1x10^{-6}$ mg/mL	$1x10^{-3}$ mg/mL	$1x10^{-4}$ mg/mL	$1x10^{-3}$ mg/mL
ISID 26	$1x10^{-2}$ mg/mL	$1x10^{-3}$ mg/mL	$1x10^{-2}$ mg/mL	$1x10^{-4}$ mg/mL	1 mg/mL
ISID 28	$1x10^{-4}$ mg/mL	$1x10^{-3}$ mg/mL	$1x10^{-5}$ mg/mL	$1x10^{-4}$ mg/mL	$1 x10^{-1}$ mg/mL
ISID 39	$1x10^{-4}$ mg/mL	$1x10^{-4}$ mg/mL	$1x10^{-3}$ mg/mL	$1x10^{-5}$ mg/mL	$1x10^{-2}$ mg/mL
ISID 45	$1x10^{-3}$ mg/mL	$1x10^{-4}$ mg/mL	$1x10^{-4}$ mg/mL	$1x10^{-6}$ mg/mL	1 mg/mL
ISID 55	$1x10^{-2}$ mg/mL	$1x10^{-3}$ mg/mL	$1x10^{-2}$ mg/mL	$1x10^{-3}$ mg/mL	$1x10^{-3}$ mg/mL

The extracts were categorised according to the substrate utilised for extraction, A— extract A using 20% wheat bran sugar cane; B— extract B using 20% wheat bran bagasse; C— extract C using 20% maize flour sugar cane; D— extract D using 20% maize flour bagasse; NPS— Zinc oxide nanoparticles.

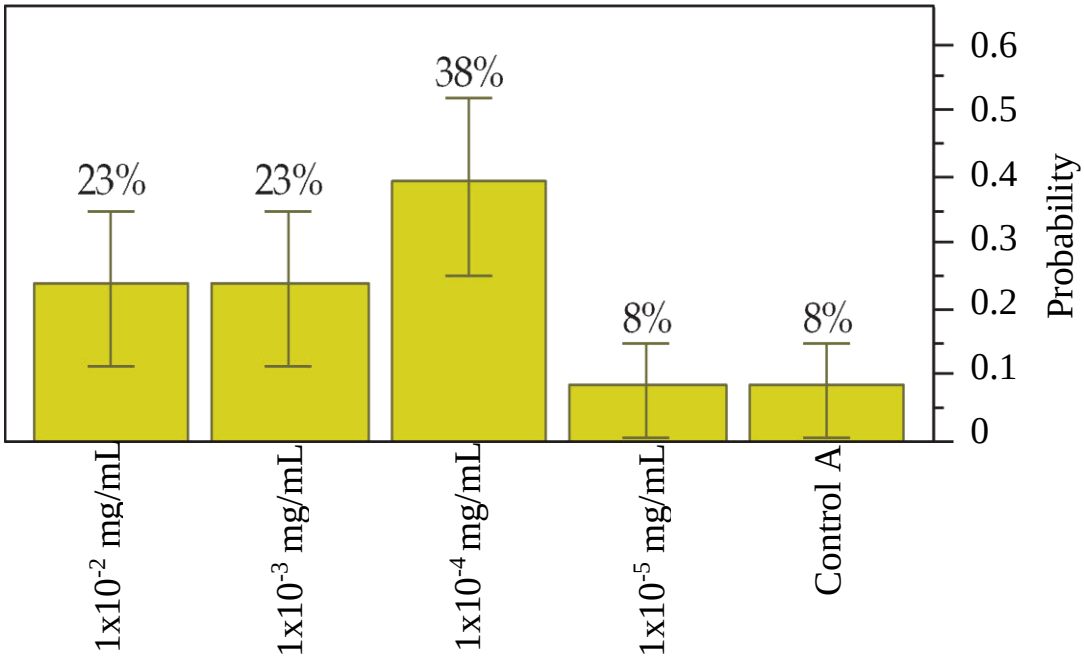

Figure 6.1: The distributions and test probabilities of extract A, extracted using 20% wheat bran sugar cane, on *N. gonorrhoeae* inhibition.

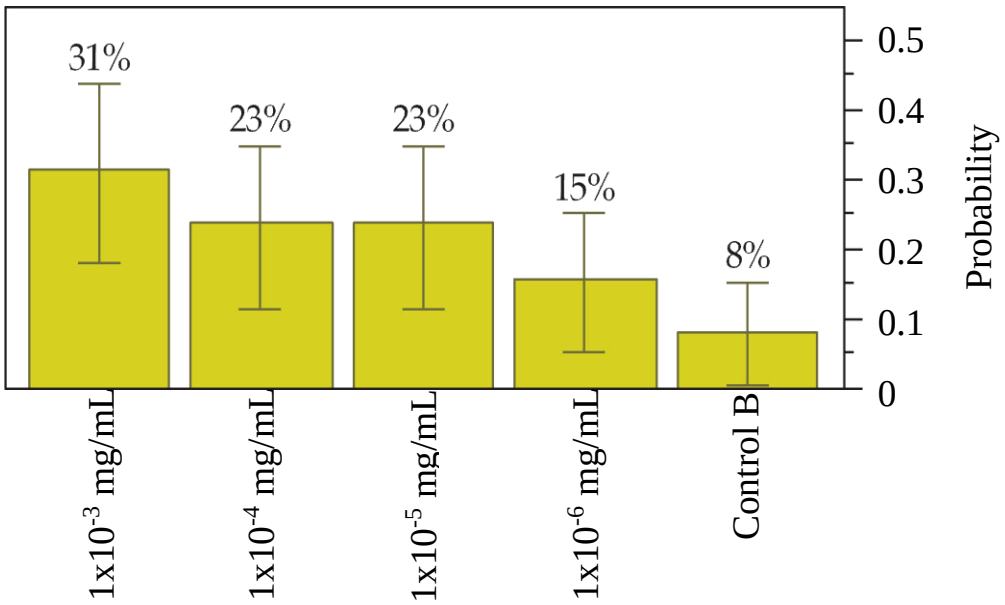

Figure 6.2: Test probabilities and distributions of extract B, extracted using 20% wheat bran bagasse, on *N. gonorrhoeae* inhibition.

In the study, the researcher found that the lowest concentration of the antibacterial agent needed to inhibit the target bacteria was observed within a range of 1×10^{-1} mg/mL and 1×10^{-5} mg/mL. The statistical analysis showed a *p*-value of 0.1539 for the lower 95% confidence interval and 0.0769 for the upper 95% confidence interval. The concentration that showed the highest inhibitory effect was 1×10^{-2} mg/mL, with a *p*-value of 0.3077. This concentration resulted in a 31% elimination rate of all targeted organisms, as depicted in Figure 6.3.

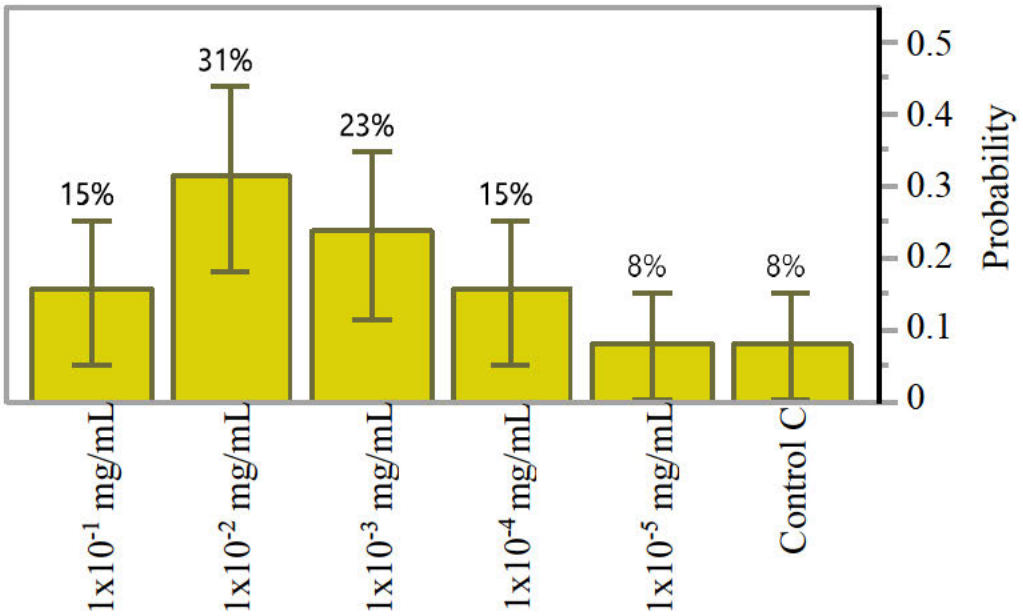

Figure 6.3: Analysis of the distributions and test probabilities of extract C, extracted using 20% maize flour sugar cane, on the inhibition of *N. gonorrhoeae*.

The concentration of extract D that showed the lowest antibacterial activity against the target bacteria ranged from 1×10^{-3} mg/mL (p = 0.0769, 95% CI: 0.0137 - 0.3331) to 1×10^{-6} mg/mL (p = 0.2308, 95% CI: 0.0818 - 0.5026). The most inhibitory concentration was observed at 1×10^{-4} mg/mL and 1×10^{-5}

mg/mL, with a *p*-value of 0.3077. The MBC elimination rate for all target organisms was 31%, as shown in Figure 6.4.

The concentration of the antibacterial agent needed to inhibit the target bacteria was found to be between 1 mg/mL and 1×10^{-3} mg/mL. The estimated probability of this concentration range was 0.1539, with a lower 95% confidence interval of 0.0433 and an upper 95% confidence interval of 0.4223. The most inhibitory concentration was observed at 1 mg/mL and 1×10^{-1} mg/mL, with an MBC elimination rate of 31% of all target organisms, as shown in Figure 6.5. A 95% confidence interval was observed, indicating a high probability of including the true value of the population parameter. Out of all the clinical isolates studied, ISID 26 and 55 showed the highest level of resistance based on MIC and MBC measurements for all extracts. Their mean MBC was around 1×10^{-2} mg/mL, respectively. The concentration of the antibacterial agent needed to inhibit the target bacteria ranged between 1 mg/mL and 1×10^{-3} mg/mL. The probability of this concentration range being effective was estimated at 0.1539, with a 95% confidence interval (CI) ranging from 0.0433 to 0.4224. This indicates that there is a 95% likelihood that the true value of the effective concentration lies within this interval. The concentration of extract D that showed the lowest antibacterial activity ranged from 1×10^{-3} mg/mL (p = 0.0769, 95% CI: 0.0137 - 0.3331) to 1×10^{-6} mg/mL (p = 0.2308, 95% CI: 0.0818 - 0.5026).

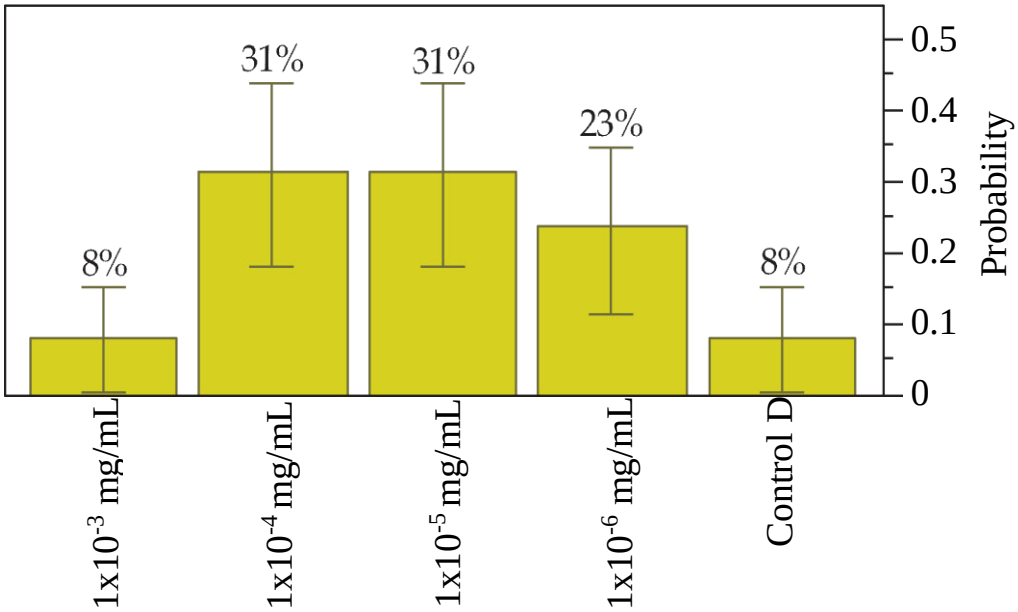

Figure 6.4: The inhibitory effects of extract D, extracted 20% maize flour bagasse, on *N. gonorrhoeae* and analysing the distributions and test probabilities.

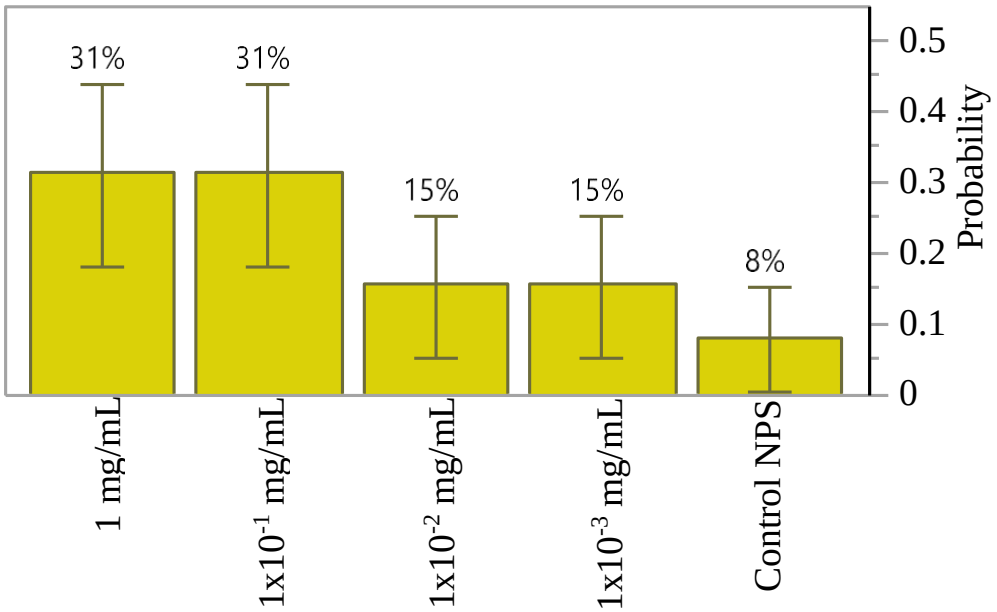

Figure 6.5: Distributions and test probabilities of Zinc oxide nanoparticles inhibitory properties against *N. gonorrhoeae*.

The most inhibitory concentration was observed at 1×10^{-4} mg/mL and 1×10^{-5} mg/mL, with a *p*-value of 0.3077, resulting in a 31% elimination rate of all target organisms, as shown in Figure 6.4. The lowest concentration needed to inhibit the target bacteria was between 1×10^{-1} mg/mL and 1×10^{-5} mg/mL. The statistical analysis showed a *p*-value of 0.1539 for the lower 95% CI and 0.0769 for the upper 95% CI, indicating a statistically significant finding. The highest inhibitory effect was seen at a concentration of 1×10^{-2} mg/mL, with a *p*-value of 0.3077, as depicted in Figure 6.3. The results show a significant difference with a *p*-value of 0.1539 (lower 95% CI: 0.0433, upper 95% CI: 0.4223). This indicates that there is a statistically significant probability that the true concentration needed for effective inhibition lies within the specified confidence intervals. The study reveals that certain antibacterial agents and mushroom extracts exhibit substantial antimicrobial properties against the target bacteria. The variations in MIC and MBC values, along with the confidence intervals and *p*-values, provide insight into the effectiveness and reliability of these agents. The findings suggest that specific concentrations, particularly those around 1 mg/mL to 1×10^{-4} mg/mL, are most effective in inhibiting and eliminating target bacteria, making these extracts promising candidates for further investigation and potential therapeutic use.

6.5 DISCUSSION

The prevalence of multidrug-resistant organisms is increasing, which is impacting the treatment of a wider spectrum of infectious diseases (Yakobi & Pooe, 2022). Therefore, there is a pressing need to uncover new and effective antimicrobial agents to counteract bacteria that have developed resistance to antibiotics (Cristillo et al., 2019). Research has shown that fungus possess remarkable potential as sources of bioactive compounds that have notable therapeutic properties. Furthermore, they are the primary generators of secondary metabolites. This paper examines the antibacterial properties of mushroom extracts against specific strains of bacteria, including ATCC strains of *S. aureus*, *E. coli*, *N. gonorrhoeae*, and nine additional clinical *N. gonorrhoeae* isolates. The extracts from *P. ostreatus*, which consisted of 20% wheat bran bagasse and 20% maize flour bagasse, had remarkable antibacterial activity against *S. aureus*, *E. coli*, and all examined *N. gonorrhoeae* isolates. Bagasse extractions were shown to have greater antibacterial activity against all species studied, making it the preferred option for extraction medium. Phenolic compounds are present in plants either as glycosides or aglycones. Due to differences in stability, they may also exist as matrix and free-bound molecules (Shi et al., 2022). Structural variations may also affect the occurrence of phenolic compounds, leading to their existence as either polymerised or monomeric forms. Phenolic compounds are unevenly dispersed throughout plants, and their stability fluctuates, which complicates the process of extraction. The efficiency of phenolic chemical recovery from samples

may be affected by both single-step extraction and poor extraction methods (Barakat et al., 2020; Vaou et al., 2021). Therefore, it is crucial to carefully choose an extraction method that is suitable for extracting the specific phenolic chemicals that are sought. The chemicals may be extracted using several methods such as conventional extraction, ultrasonic-assisted extraction (UAE), reflux extraction, microwave-assisted extraction (MAE), soxhlet extraction, supercritical fluid extraction (SFE), and pulsed electric field extraction (PEF). During the process of extracting sugar from sugarcane, about 25% of the total sugarcane processed results in the production of a remnant biomass called bagasse. Bagasse has a significant calorific value and has lately been used for the production of biogas due to its composition of hemicellulose, cellulose, lignin, and soluble sugars. Nevertheless, the profitability of producing biogas from bagasse is hindered by its resistant characteristics. Consequently, several pre-treatment methods are being explored to decrease the recalcitrance of the lignin-protected substrate. The findings suggest that the use of bagasse in mushroom extractions enhances the extraction of bioactive compounds, leading to heightened antibacterial activity against at least three bacterial species examined in this work. Mushroom extracts exhibited higher levels of bioactive components when mixed with 20% wheat bran bagasse and 20% maize flour bagasse, compared to extracts containing 20% wheat bran sugarcane and 20% maize flour sugarcane. Within this particular framework, the results are analogous to the findings of previous studies on the

antimicrobial properties of indigenous mushrooms on different types of human diseases.

Several antimicrobial phenolic compounds, such as *p*-OH-Benzoic acid, *p*-OH-Phenylacetic acid, protocatechuic acid, and syringic acid, among others, may be generated during the extraction process. These findings align with a study on the antibacterial properties of the entire fruiting bodies of *P. ostreatus* against different types of fungi. The study revealed that both the fruiting bodies and mycelia obtained from laboratory cultures have the ability to generate compounds that could be used in medicine, pharmacology, and cosmetics. Other investigations have also shown the antimicrobial effects of mushroom extracts obtained from different solvents. According to the research, the methanolic, ethanolic, and aqueous extracts of *P. ostreatus* were shown to inhibit the development of *E. coli* cultures, indicating their antibacterial properties (Gashaw et al., 2020). In another research, the antibacterial characteristics of *P. ostreatus* extracts against *S. aureus* were examined. The study indicated that petroleum ether and acetone extracts of *P. ostreatus* were efficient against Staphylococcus spp. (Miklasińska-Majdanik et al., 2018; Park & Seo, 2022). Therefore, the variation in antibacterial activity of *P. ostreatus* against *S. aureus* may be attributed to differences in the geographical locations of the mushroom's habitats and the substrates utilised for extraction. Recent research conducted a comparative analysis of the antibacterial efficacy of mushroom extract on Gram-

negative and Gram-positive bacteria. The analysis revealed that the extracts exhibited more efficacy in suppressing the growth of Gram-negative bacteria compared to Gram-positive bacteria. The differential in sensitivity to antibiotic extracts between Gram-positive and Gram-negative bacteria has been associated with morphological differences in many investigations (Alves et al., 2012; Matijaševic et al., 2016; Ramesh & Pattar, 2010). The presence of lipopolysaccharide in the outer membrane of Gram-negative bacteria is believed to make the cell wall impermeable to lipophilic extracts. This opens up opportunities for further investigation into the already studied *P. ostreatus* extracts against a broader spectrum of Gram-positive and Gram-negative bacteria. The present investigation could not find any statistically significant difference between Gram-positive and Gram-negative species.

Studies have shown that different mushroom extracts and extraction methods had distinct antibacterial effects against both Gram-positive and Gram-negative bacteria (Kosanić et al., 2012; Matijaševic et al., 2016). The findings align with previous studies on the antibacterial activities and mineral contents of different mushrooms cultivated on agricultural waste. In this research, it has been shown that *S. aureus*, a kind of Gram-positive bacteria, had a high level of sensitivity and was inhibited by 20% wheat bran bagasse extractions. This inhibition was compared to all *N. gonorrhoeae* clinical isolates and other ATCC strains that were included in the analysis. Recent study has shown that both chloroform and water

extracts of mushrooms had strong antibacterial activity against *S. aureus* compared to other Gram-negative bacteria studied. When examining the clinical isolates, it was found that the wheat bran bagasse extracts had the strongest antibacterial effect against the *N. gonorrhoeae* organisms, with a potency of 20%. Comparing this to the antibacterial effects of the five antibiotics studied, it was observed that the two isolates, ISID 26 and ISID 55, which showed the most resistance to the antibiotics during screening, also had the highest minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) compared to the other clinical isolates. These data suggest that the amount of *P. ostreatus* extract required to successfully inhibit the development of a particular bacterium grows in direct proportion to the organism's level of antibiotic resistance and the range of microorganisms it may affect. The study investigated the varying degrees of antibacterial activity in *P. ostreatus* extracts within the same type of organisms. The differences in the antibacterial effectiveness of the extracts may be due to factors that contribute to resistance, such as conjugative plasmids, transposons, and insertion sequences. Moreover, the variations in antimicrobial effectiveness can be ascribed to various factors, such as the genetic makeup of the clinical isolates, which can lead to changes in the physical and biochemical characteristics of the organism due to mutations. Another possible reason for these differences could be the type of substrate used in the extraction of bioactive agents. The reduced antibacterial activity of the 20% Wheat Bran Sugar Cane and 20% Maize Flour Sugar Cane extracts may be attributed to the

absence of significant secondary metabolites, which is a result of the effectiveness of the extraction procedure. The extracts exhibited diverse MIC antimicrobial activities against *E. coli* and *S. aureus*, with an average extract concentration of $1x10^{-5}$ mg/mL. However, *N. gonorrhoeae* ATCC 49926 showed a higher MIC mean concentration of $1x10^{-3}$ mg/mL, indicating that *N. gonorrhoeae* has a greater resistance to the target extracts compared to *E. coli* and *S. aureus*.

All the extracts tested in this study have shown some degree of effectiveness against both the clinical isolates and standard strains. Nevertheless, most of the bacteria obtained from clinical sources have shown greater resistance to the extracts compared to the conventional *N. gonorrhoeae* strain. The main factor is the unselective exposure of clinical isolates to numerous antimicrobial medications.

6.6CONCLUSION

The current study highlights the potential of polyphenols as a promising source of effective, safe, and affordable antimicrobial compounds. The wide range of natural compounds with antimicrobial properties offers numerous possibilities for developing new antibacterial treatments to combat multidrug-resistant clinical isolates. It is important to note that this study exclusively examined the effects of these compounds in a laboratory setting. Because of their limited effectiveness as standalone treatments, polyphenols with higher MICs than current antibiotics

cannot be utilised as antimicrobial monotherapy. However, when used in combination with antibiotics, these polyphenols may improve the pharmacokinetic and pharmacodynamic properties. In addition, the utilisation of polyphenols has the potential to reduce medication dosages and mitigate the negative side effects of antibiotics. In order to assess the effectiveness of these antibacterial drugs in a clinical setting, it is crucial to prioritise further research on in vivo experiments and clinical trials.

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CHAPTER 7: ANTIMICROBIAL POTENTIAL OF ORGANIC PHENOLIC COMPOUNDS FROM *P. OSTREATUS* EXTRACTS: IMPACT ON PROLIFERATION AND KINETIC GROWTH OF CLINICAL MULTI-DRUG RESISTANT *NEISSERIA GONORRHOEAE* STRAINS

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Preface

This article delves into the antimicrobial potential of organic phenolic compounds derived from mushroom extracts. The artiled aimed to investigate the bioactivity impact of these compounds on the proliferation and kinetic growth of multi-drug resistant strains of *Neisseria gonorrhoeae*. This research article was published with the Journal of Food Biochemistry, on August 3, 2024. DOI link: https://doi.org/10.1155/2024/2336255.

7.1ABSTRACT

Extracts derived from various mushroom species have been documented to possess notable antimicrobial properties. However, the current corpus of knowledge pertaining to the precise evaluation of their structural characteristics is currently inadequate. In this study, a comprehensive analysis was undertaken to ascertain the antimicrobial attributes and effectiveness of phenolic compounds identified from *P. osteatus*. These compounds were examined for potential anti-proliferation properties against multidrug-resistant gonococcal clinical isolates. The results of the study revealed that *p*-hydroxybenzoic acid, *o*-coumaric acid, and chysin exhibited no antibacterial activity (MIC > 50 µg/mL) against any of the target *N. gonorrhoeae* isolates in the range of tested concentrations (0.1–50 µg/mL). A notable reduction in the growth activity of the target organisms was observed when subjected to cultivation in the presence of flavonoid compounds. The statistical significance of the parameter estimate for quercetin was observed at Intercept [ISID 59], with a *p*-value of less than 0.0001 and a ChiSquare value of 44.84. The combination of ferulic acid with either protocatechuic acid or *p*-coumaric acid produced a noticeable trend towards ineffective antimicrobial action against most of the target isolates. However, the findings highlight its remarkable promise, as quercetin exhibited both independent and cooperative effectiveness, thereby illustrating its vast potential for future investigations with a notable emphasis on safety.

KEYWORDS

Phenolic Acids, Flavonoids, Growth Kinetics, Pharmacokinetic Properties, Antibacterial Activity, Multidrug-Resistant *Neisseria gonorrhoeae*

7.2INTRODUCTION

Antimicrobial susceptibility profiles and global trends of *N. gonorrhoeae* indicate a high prevalence of resistance to penicillin, tetracycline, and ciprofloxacin. This resistance significantly limits the use of these drugs for empirical treatment recommendations (Chen et al., 2019; Mabonga et al., 2019; Meyer and Buder, 2020). Rising azithromycin resistance rates have also been documented globally, which is concerning given that azithromycin is still part of the current recommended combination treatment for gonococcal infections (Jefferson *et al.*, 2021; Unemo *et al.*, 2021). Along with rising moderate-level azithromycin resistance, there have been reports of high-level azithromycin resistance with a MIC greater than 256 mg/L (Salmerón et al., 2020). The emergence of multidrug-resistant (MDR) *N. gonorrhoeae* strains has been well documented by surveillance programmes all around the world (Unemo, 2015; Bodie et al., 2019). These strains present a significant treatment challenge, and to overlap the limitations of the available antimicrobial drugs for these strains, novel drugs with new mechanisms of action are synthesised (Terreni et al., 2021). Recently, there have been an increasing number of reports on phenolic compounds identified from different mushroom species that have been found to have significant

antibacterial activity (Fakoya et al., 2020; Cardoso et al., 2021). These compounds have been shown to have antiviral, anticancer, antibiotic, and antibacterial properties (Patra, 2012; Anusiya et al., 2021). Although the mechanisms of antibacterial activity of these phenolic compounds are not completely understood, it has been suggested that these compounds involve several sites of action at the cellular level (Miklasińska-Majdanik et al., 2018; Vaou et al., 2021). Several researchers explained this activity by the modification of the permeability of cell membranes, changes in various intracellular processes produced by hydrogen binding of phenolic compounds to enzymes, or a change in cell wall rigidity with integrity losses owing to diverse interactions with the cell membrane (Bouarab Chibane et al., 2019; Ghendov-Mosanu et al., 2022). Therefore, increasing the lipophilicity of phenolic compounds improves their antibacterial activity by facilitating their contact with the cell membrane (Miklasińska-Majdanik et al., 2018). It was further suggested that this may also lead to permanent cytoplasmic membrane damage and coagulation of cell content, which can even impede intracellular enzymes. Condensed phenylpropanoids—tannins, have been demonstrated to disrupt membrane integrity of Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, and *Listeria monocytogenes*) (Bouarab-Chibane et al., 2019), while phenolic acids have been reported to disrupt membrane integrity in Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enteritidis*) and cause leakage of important intracellular contents (Makarewicz et al., 2021; Ecevit

et al., 2022). A recent study showed that flavonoids may interact with soluble proteins found outside bacterial cell walls, facilitating the creation of complexes (Donadio et al., 2021). It was further noted that flavonoids may also potentially impact protein and RNA production by decreasing both energy consumption and DNA synthesis (Kumar and Pandey, 2013). Flavonoids are a significant group of polyphenolic compounds with a benzo-pyrone structure that are abundant in plants. They are produced through the phenylpropanoid pathway (Panche et al., Chandra, 2016). Numerous pharmacological activities are attributed to secondary metabolites of phenolic nature, including flavonoids, according to the available evidence (Tungmunnithum et al., 2018). Flavonoids are hydroxylated phenolic compounds that are produced by plants in response to microbial infection (Roy et al., 2022). Flavonoids' chemical constitution is determined by their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization (Sarian et al., 2017). The potential health benefits resulting from the antioxidant properties of these polyphenolic compounds have sparked a recent interest in these substances (Singh & Yadav, 2022). By scavenging free radicals and/or by chelating metal ions, functional hydroxyl groups in flavonoids mediate their antioxidant effects (Kumar & Pandey, 2013). Chelation of metals may be essential for the prevention of radical generation that damages biomolecules of interest. In this context, the aim of this study was to determine the antibacterial activity of the selected phenolic compounds, quercetin, rutin, *p*-coumaric acid, ferulic acid, and protocatechuic acid, and evaluate the modulatory

effect of these compounds on the proliferation of *N. gonorrhoeae* clinical isolates exhibiting a diverse range of antimicrobial resistance.

7.3MATERIALS AND METHODS

Please refer to chapter 3, subsection 3.5 – 3.7 and 3.12, page 104 – 108 and 117 – 118 repsectively, for a detailed material and methods protocol.

7.4RESULTS AND DISCUSSION

7.4.1 Antibacterial Assay Compounds Derived from Mushroom Extract

Potential antibacterial compounds were identified through compound extraction. However, none of the tested concentrations (0.1–50 µg/mL) of *p*-hydroxybenzoic acid, *o*-coumaric acid, and chysin demonstrated antibacterial activity (MIC > 50 µg/mL) against any of the target *N. gonorrhoeae* isolates. A multitude of environmental factors, such as the various techniques employed to extract phenolic compounds and the diverse enzymatic and oxidative degradation that occurs after collection, have been recognised as influencing factors in both the quantity and quality of the compounds (Bouarab Chibane et al., 2019; Miklasińska-Majdanik et al., 2018). The phenolic compounds extracted from *P. ostreatus* and evaluated for their potential to eradicate various strains of *N. gonorrhoeae* bacteria are presented in Table 7.1. In addition to their well-known antioxidant properties, these compounds have exhibited antibacterial activity, suggesting they may be capable of countering a wide range of resistance

mechanisms. Susceptibility to the compounds is correlated with the susceptibility profiles of the target microorganisms, as indicated by the measured MIC values.

Table 7.1: Analysis of the MIC values (in micrograms per millilitre) of several phenolic compounds against clinical isolates of *Neisseria gonorrhoeae*.

Compounds	**MIC range (µg/mL)**	**Mean range (µg/mL)**
Phenolic Acids:		
Gallic acid	5<conc<50 µg/mL	27.5 µg/mL
p-Hydroxybenzoic acid	≥50 µg/mL	50 µg/mL
Protocatechuic acid	2<conc<50 µg/mL	17.4 µg/mL
Cannamic Acids:		
Ferulic acid	1 – 20 µg/mL	8.6 µg/mL
o-Coumaric acid	>50 µg/mL	65.3 µg/mL
p-Coumaric acid	0.5<conc<50 µg/mL	15.1 µg/mL
Flavonoids:		
Quercetin	0.5 – 5 µg/mL	1.8 µg/mL
Rutin	1 – 10 µg/mL	3.6 µg/mL

The MIC values indicate the concentration at which the phenolic compounds hinder the growth of the target bacteria. Phenolic acids, specifically gallic acid, exhibited a range of MIC values from 5 to less than 50 µg/mL, with an average MIC value of 27.5 µg/mL. These findings indicate that the treatment was

successful in combating the different strains to different extents, resulting in an overall moderate level of effectiveness. However, the *p*-hydroxybenzoic acid exhibited MIC values of 50 µg/mL or higher. This resulted in an average MIC value of 50 µg/mL, indicating that it displayed relatively lower antimicrobial activity against the tested isolates. Finally, the MIC range for protocatechuic acid is 2 to less than 50 µg/mL, with an average MIC value of 17.4 µg/mL, indicating a moderate level of inhibitory potential. Regarding cannamic acids, ferulic acid demonstrated a wide range of MIC values, ranging from 1 to 20 µg/mL. On average, the MIC value was determined to be 8.6 µg/mL, indicating significant antimicrobial effectiveness against the various isolates. Although the MIC values of *o*-coumaric acid are higher than 50 µg/mL, with a mean MIC value also higher than 50 µg/mL, this compound showed limited effectiveness against the tested isolates. The MIC range of *p*-Coumaric acid spanned from 0.5 to over 50 µg/mL, resulting in an average MIC value of 15.1 µg/mL. This indicates that the activity of *p*-Coumaric acid varied, with certain isolates displaying greater susceptibility. Flavonoids have proven to be highly effective against the target clinical isolates. In particular, quercetin has shown remarkable antimicrobial activity, with MIC ranges ranging from 0.5 to 5 µg/mL. This translates to a mean MIC value of 1.8 µg/mL, demonstrating its potent antimicrobial activity across all the isolates. In addition, rutin exhibited a wide range of MIC values, ranging from 1 to greater than 10 µg/mL. On average, the MIC value was found to be 3.6 µg/mL. This indicates that rutin's effectiveness varied among different isolates. The results of

this study emphasise the diverse antimicrobial capabilities of various phenolic compounds when tested against clinical isolates of *Neisseria gonorrhoeae*. Certain compounds, like quercetin, rutin, and ferulic acid, have shown strong inhibitory activity with a mean MIC range of less than 10 µg/mL. On the other hand, *o*-coumaric acid and *p*-hydroxybenzoic acid have demonstrated more limited effects, with a MIC of 50 µg/mL or higher. The differences in MIC values highlight the intricate nature of bacterial reactions to various phenolic compounds and their potential use in fighting against drug-resistant *Neisseria gonorrhoeae* infections. It is worth mentioning that the variations in the findings reported by multiple researchers may be attributed to the utilisation of strains with varying resistance profiles, as well as the use of different methodologies. These methodologies encompass the use of different solvents for preparing compound solutions and diverse techniques for determining minimum inhibitory concentrations (MICs). In this study, water was selected as the safest solvent option. However, for flavonoids, a solution of water with 1% DMSO was utilised to ensure complete solubility of the compounds. There has been growing concern about the global prevalence of multidrug-resistant *N. gonorrhoeae*, which has become a major medical issue in recent years. There is a concerning issue that lurks in the realm of treatment failure and resistance development, where the probability of superbug evolution looms. The data obtained in this study suggest that certain compounds, such as quercetin, may possess properties that could potentially inhibit and treat multidrug-resistant strains.

The study identified *p*-hydroxybenzoic acid, *o*-coumaric acid, and chrysin as phenolic compounds extracted from *P. ostreatus* that did not exhibit antibacterial activity against the tested *N. gonorrhoeae* isolates at concentrations ranging from 0.1 to 50 µg/mL. This finding suggests that these specific compounds, under the conditions tested, do not possess antimicrobial properties effective against *N. gonorrhoeae*. The results further highlight the influence of various environmental factors on the quantity and quality of phenolic compounds extracted from *P. ostreatus*. Factors such as extraction techniques and enzymatic/oxidative degradation post-collection can significantly affect the bioactivity of extracted compounds. This underscores the complexity of natural product research and the importance of considering extraction methodologies and environmental influences in experimental design. Despite the non-activity of *p*-hydroxybenzoic acid, *o*-coumaric acid, and chrysin against *N. gonorrhoeae*, the study notes that other phenolic compounds extracted from *P. ostreatus* have demonstrated antibacterial activity. Furthermore, these compounds are recognized for their antioxidant properties, which are beneficial for overall health and may contribute to their potential therapeutic effects. The observed susceptibility of *N. gonorrhoeae* strains to the tested phenolic compounds correlates with their susceptibility profiles, as indicated by the MIC values obtained. This correlation suggests that the antimicrobial activity of these compounds is specific to the microbial strains tested and may vary based on strain-specific resistance mechanisms.

The study found specific compounds such as ferulic acid, quercetin, and rutin, which demonstrated strong inhibitory potential with average MIC values below 10 µg/mL. Conversely, compounds like *p*-hydroxybenzoic acid and o-coumaric acid displayed limited effectiveness, with MIC values consistently at or above 50 µg/mL, suggesting weaker antimicrobial activity against target *N. gonorrhoeae* isolates. These findings underscore the potential of certain phenolic compounds, particularly quercetin, to combat drug-resistant *N. gonorrhoeae* infections. Their ability to inhibit bacterial growth at low concentrations suggests they could be explored further as therapeutic agents against multidrug-resistant strains, addressing an urgent medical need amid rising concerns of treatment failure and antibiotic resistance. The study acknowledges the influence of methodological factors, such as solvent choice (water vs. water with 1% DMSO) and variability in strain resistance profiles, on reported MIC values. This highlights the importance of standardized methodologies in antimicrobial research to ensure reliable comparisons across studies. However, given the global prevalence of multidrug-resistant *N. gonorrhoeae* and the limited treatment options available, the identification of effective phenolic compounds provides promising avenues for developing alternative treatments. Compounds like quercetin, with potent antimicrobial properties, offer hope in combating the evolving challenge of antibiotic-resistant infections.

7.4.2 SWISSADME

The SwissADME software was employed to evaluate the structural characteristics and bioavailability of candidate compounds with potential antimicrobial activity against *N. gonorrhoeae*. Analysis of the compounds' physicochemical properties, including lipophilicity, solubility, and pharmacokinetics, provided valuable insights into their potential efficacy and suitability as antimicrobial agents. The lipophilicity (LogP) predictions from SwissADME are crucial as they influence the compound's ability to penetrate *N. gonorrhoeae* cell membranes. Compounds with optimal LogP values may exhibit enhanced membrane permeability, potentially improving their ability to reach intracellular targets and exert antimicrobial effects. Moreover, predictions of aqueous solubility are pivotal for understanding the compound's bioavailability and effectiveness in aqueous environments such as bodily fluids. Higher solubility may enhance the compound's distribution and availability at infection sites, thereby impacting its antimicrobial potency against *N. gonorrhoeae*. Additionally, insights from pharmacokinetic predictions provided by SwissADME, such as absorption, distribution, metabolism, and excretion (ADME) properties, offer critical considerations. Compounds predicted to have favourable ADME profiles are more likely to achieve therapeutic concentrations *in vivo*, crucial for combating *N. gonorrhoeae* infections effectively. Furthermore, drug likeness assessments from SwissADME inform about the compound's similarity to known drugs and its potential for clinical development.

Compounds meeting drug likeness criteria are more likely to progress through subsequent stages of drug discovery and may represent promising candidates for treating *N. gonorrhoeae* infections.

The concentrations of the tested compounds were meticulously chosen through a systematic approach. At first, some initial experiments were carried out, where the compounds were tested using a six-fold dilution series. The dilutions were carried out to determine the approximate MIC values for each specific agent. Physicochemical descriptors to assist in predicting the ADME parameters, pharmacokinetic properties, drug-likeness, and medicinal chemistry compatibility of one or more small molecules was used, see Table 7.2. The molecular weight of rutin is 610.52 g/mol. There are a total of 43 heavy atoms in the composition, with 16 of them being aromatic heavy atoms. The calculated fraction of sp3-hybridized carbon atoms is 0.44. This compound has a total of six rotatable bonds. In addition, it has 16 hydrogen bond acceptors and 10 hydrogen bond donors. The molar refractivity of rutin is found to be 141.38, while it has a total polar surface area (TPSA) of 269.43 Å. This compound does not have the ability to pass through the blood-brain barrier, which is a selective barrier. This substance acts as a substrate for *P*-glycoprotein, a transporter protein that plays a role in the efflux of different substances. In addition, it does not show any inhibitory effects on the enzymes that play a crucial role in metabolising various drugs. Finally, the skin permeation coefficient (Log Kp) for this compound is

calculated to be -10.26 cm/s, suggesting a significantly slow rate of permeation through the skin. Quercitin is a flavonoid compound that has a molecular weight of 302.24 g/mol. Quercetin contains no sp3 hybridised carbon atoms (Csp3). The molecule has one rotatable bond and has a total of seven hydrogen bond acceptors and five hydrogen bond donors. The molar refractivity of quercetin is determined to be 78.03, while its total polar surface area (TPSA) measures 131.36 Å. This substance does not easily pass through the blood-brain barrier, does not exhibit the characteristics of a *P*-glycoprotein substrate, and can inhibit the cytochrome P450 1A2 enzyme. This particular compound has been found to have an impact on the enzymatic isoforms CYP2D6 and CYP3A4. Nevertheless, it does not demonstrate any inhibitory effects on the activities of CYP2C19 and CYP2C9. In addition, the log Kp value, which indicates the rate of skin permeation, is measured at -7.05 cm/s. The molecular weight of protocatechuic acid is 154.12 g/mol. This compound has a total of 11 heavy atoms, with 6 of them being aromatic heavy atoms. The determined fraction of sp3-hybridized carbon atoms, denoted as Csp3, is 0.00. This compound has a single rotatable bond. In addition, it has 4 hydrogen bond acceptors and 3 hydrogen bond donors. the research indicates that protocatechuic acid has a total polar surface area (TPSA) of 77.76 Å and a molar refractivity of 37.45. This particular compound does not have the ability to pass through the blood-brain barrier (BBB) and instead serves as a substrate for *P*-glycoprotein (*P*-gp). There are no inhibitory effects observed on the enzymes CYP1A2, CYP2C19, CYP2C9, or CYP2D6. Nevertheless, it does

hinder the activity of the enzyme CYP3A4. In addition, it has a logarithmic skin permeation coefficient (Log Kp) of -6.42 cm/s. Ferulic acid has a molecular weight of 194.18 g/mol. The composition includes 14 heavy atoms, with six of them having aromatic properties. The proportion of sp3 hybridised carbon atoms, represented as Csp3, is 0.10. This compound has three rotatable bonds. In addition, it has 4 hydrogen bond acceptors and 2 hydrogen bond donors. The molar refractivity of ferulic acid is determined to be 51.63, while its total polar surface area (TPSA) measures 66.76 Å. The compound being studied demonstrates the ability to pass through the blood-brain barrier (BBB), suggesting it can traverse this protective barrier. In addition, it does not act as a substrate for *P*-glycoprotein (*P*-gp), a transporter protein that plays a role in removing different compounds from cells. It is crucial to understand that this compound does not inhibit the function of certain cytochrome P450 enzymes (CYPs), namely CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4. Cytochrome P450 enzymes are a large and diverse group of heme-thiolate proteins that play crucial roles in the metabolism of various substrates. In bacteria, particularly *N. gonorrhoeae*, these enzymes are involved in a range of biochemical processes essential for survival and pathogenicity. The primary function of bacterial CYPs is to catalyse the oxidation of organic substrates, a reaction that often introduces an oxygen atom into the substrate, leading to the formation of a more hydrophilic product. This oxidative capability is particularly important in the metabolism of endogenous compounds, such as fatty acids and

steroids, as well as xenobiotics, including antibiotics and other antimicrobial agents. CYPs contribute to the organism's ability to evade the host immune system and adapt to various environmental stresses. One of the critical roles of CYPs in *N. gonorrhoeae* is in the modification and detoxification of host-derived molecules and antimicrobial agents, which can enhance the bacterium's survival and persistence in the host (Kirkcaldy et al., 2019; Martin et al., 2016). Moreover, CYPs are involved in the biosynthesis of essential cell membrane components and signalling molecules that are vital for maintaining cell integrity and mediating interactions with the host environment. The biological role of CYPs in *N. gonorrhoeae* extends to their participation in the metabolic pathways that enable the bacterium to utilize various nutrient sources efficiently. This adaptability is crucial for colonization and infection, as *N. gonorrhoeae* often encounters fluctuating nutrient availability within different host tissues (Taylor et al., 2018). Furthermore, the activity of CYPs in modifying signalling molecules can influence the regulation of gene expression, contributing to the bacterium's ability to respond to stress and antibiotic exposure.

The logarithmic skin permeation coefficient (Log Kp) has been calculated to be -6.41 cm/s, which suggests that it has the capacity to penetrate the skin. *p*-Coumaric acid is a compound with a molecular weight of 164.16 g/mol.

All the target compounds have been thoroughly validated based on various criteria for drug-likeness, with the exception of rutin. The process of compound

absorption in the gastrointestinal (GI) tract is highly efficient, leading to a substantial level of bioavailability. The partition coefficient (Log Po/w or iLOGP) for these compounds varies from 0.66 to 1.63, as observed. In addition, the water solubility of these compounds is essential for evaluating how they are absorbed and distributed within the human body. The water solubility values, as shown in Table 7.2, range from 2.11e-01 mg/mL to 1.58e+00 mg/mL. The observed pharmacokinetic parameters indicate that the ligand in question exhibits characteristics commonly seen in traditional drug molecules, as they fall within the accepted range.

After analysing their bioavailability scores and lead likeness, it has been determined that quercetin, protocatechuic acid, *p*-coumaric acid, and ferulic acid exhibit more favourable drug likeness profiles compared to rutin. When choosing compounds for drug development, it is crucial to take into account these properties. They have a significant influence on the effectiveness and potential of the compound as a therapeutic agent.

Table 7.2: Analysed data on molecular properties of target phenolic compounds to evaluate their potential for drug development and bioavailability.

Compound	**Log Po/w (iLOGP)**	**Solubility**	**GI absorption**	**Drug likeness (Lipinsk)**	**Bioavailability Score**	**Lead likeness**
Rutin	1.58	3.08e-01 mg/mL ; 5.05e-04 mol/l (Soluble)	Low	No; 3 violations: MW>500, NorO>10, NHorOH>5	0.17	No; 1 violation: MW>350
Quercetin	1.63	2.11e-01 mg/mL ; 6.98e-04 mol/l (Soluble)	High	Yes; 0 violation	0.55	Yes
Protocatechuic acid	0.66	2.14e+00 mg/mL ; 1.39e-02 mol/l (Very soluble)	High	Yes; 0 violation	0.56	No; 1 violation: MW<250
***p*-Coumaric acid**	0.95	1.58e+00 mg/mL ; 9.65e-03 mol/l (Soluble)	High	Yes; 0 violation	0.85	No; 1 violation: MW<250
Ferulic acid	1.62	1.49e+00 mg/mL ; 7.68e-03 mol/l (Soluble)	High	Yes; 0 violation	0.85	No; 1 violation: MW<250

7.4.3 Kinetic growth assay

The growth rates of the target isolates showed significant variation. It is worth mentioning that the *N. gonorrhoeae* isolates exhibited a more rapid growth rate when exposed to *p*-coumaric acid. There was a decrease in growth activity when the target organisms were cultivated in the presence of flavonoid compounds. Multiple scientific studies have examined the effectiveness of extracts rich in flavonoids and isolated flavonoid compounds in inhibiting the growth of specific harmful bacteria. Flavonoids have attracted considerable interest because of their possible antibacterial properties (Liu et al., 2019). Several mechanisms have been proposed to explain the complex actions that underlie the antibacterial effects of flavonoids. Flavonoids function by creating a complex with the proteins found in the cellular wall. Research has shown that certain measures can effectively prevent the attachment and proliferation of microorganisms. Based on additional research, it has been found that the way this works is by blocking certain bacterial enzymes, particularly tyrosyl tRNA synthetase (Faleye et al., 2023). According to the theory, flavonoids possess a range of antibacterial effects. They have the ability to disrupt the production of nucleic acids, alter the functioning of the cytoplasmic membrane, hinder energy metabolism, prevent cell aggregation and biofilm formation, impede the functioning of porin on the cell membrane, modify membrane permeability, reduce pathogenicity, and cause damage to the cytoplasmic membrane (Faleye et al., 2023).

The isolates showed a remarkable ability to adapt to *p*-coumaric acid and ferulic acids, surpassing their adaptability to protocatechuic acid. Interestingly, ferulic acid did not have a significant impact on growth promotion, especially in ISID 46, while *p*-coumaric acid had the smallest effect on the growth rate of ISID 54. According to recent research, *p*-coumaric is believed to work by inducing permanent changes in the cell membrane, leading to the loss of the cell's ability to maintain important molecules within the cytoplasm. Additionally, it has been found to bind to DNA, effectively hindering various cellular functions. Based on the statistical analysis, the action of protocatechuic acid showed an average value of 7.19, with a standard deviation of 0.25 (refer to Figure 7.1). A value of 0.08 was determined as the standard error of the mean. The mean was determined to have an upper 95% confidence interval of 7.37 and a lower 95% confidence interval of 7.01.

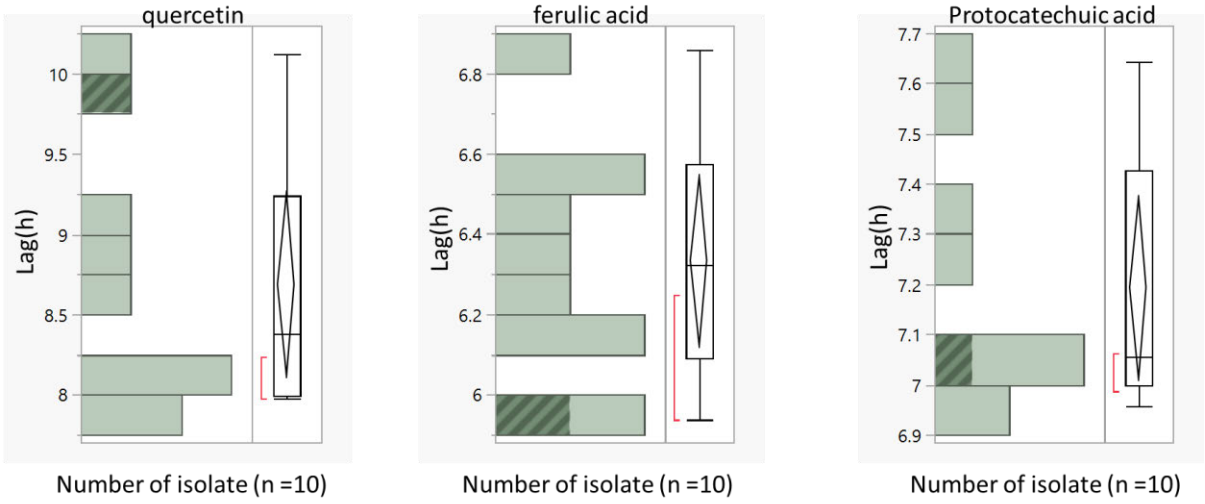

Figure 7.1: The distribution statistics related to the effects of quercetin, ferulic acid, and protocatechic acid.

In Figure 7.1, it can be observed that ferulic acid, a phenolic compound commonly present in plants, showed an average value of 6.33, with a standard deviation of 0.29. The calculated standard error of the mean is 0.0928213. The mean was determined to have an upper 95% confidence interval of 6.54 and a lower 95% confidence interval of 6.1231237. Similarly, *p*-coumaric acid, another phenolic compound found in different plant sources, showed an average value of 4.51. The calculated standard deviation for *p*-coumaric acid is 0.42, while the standard error of the mean is determined to be 0.13. The estimated upper 95% confidence interval for the mean was 4.8163451, while the lower 95% confidence interval was found to be 4.21. The flavonoid compound Quercetin displayed an average value of 8.684, accompanied by a standard deviation of 0.79 (refer to Figure 7.1). Based on the data analysis, the standard error of the mean was found to be 0.25. Additionally, the upper 95% confidence interval for the mean was determined to be 9.2526295. The calculated lower 95% mean value of the provided data is 8.1153705. The experimental data for rutin showed an average value of 12.46, with a standard deviation of 1.12. A value of 0.35 was determined as the standard error of the mean. In addition, the mean was calculated to have an upper 95% confidence interval of 13.26 and a lower 95% confidence interval of 11.65.

A thorough evaluation of the antimicrobial activity of all compound samples was carried out, producing significant findings. The reduced model showed a log

likelihood value of 23.02, whereas the full model had log likelihood values for different acids. These findings offer valuable insights into the antimicrobial properties of the mentioned compounds. For the combination of protocatechuic acid, ferulic acid, and rutin, the model's Difference in Log-Likelihood values were observed to be 23.02. Similarly, *p*-coumaric acid had a value of 22.397654, while quercetin had a value of 21.525317. The degrees of freedom (DF) were calculated to be 9, while the ChiSquare values obtained were 46.05 for protocatechuic acid, ferulic acid, and rutin, 44.79 for *p*-coumaric acid, and 43.05 for quercetin. It is worth mentioning that the Prob>ChiSq (*p*-value, is a measure used in statistical hypothesis testing to determine the significance of results obtained from a chi-square test) for all these compounds was determined to be less than 0.0001, suggesting a highly significant statistical association. The coefficient of determination (R2) was calculated to be 1.0 for protocatechuic acid, ferulic acid, and rutin, suggesting an excellent fit. The R2 value for *p*-coumaric acid showed a strong correlation with a value of 0.97. Similarly, quercetin showed a significant correlation with an impressive R2 value of 0.93. The BIC values for protocatechuic acid, ferulic acid, rutin, *p*-coumaric acid, and quercetin were determined to be 41.44, 42.70, and 44.45, respectively. Based on the data, it is evident that the observed correlations are statistically significant. The parameter estimates for protocatechuic acid showed a strong statistical significance at the intercept. The *p*-value was less than 0.0001 and the ChiSquare value was 36.10. The statistical significance of the parameter estimate for ferulic acid was found

to be at the intercept, with a value of 0.0002 and a corresponding ChiSquare of 14.36. Based on the analysis, it was found that there was no statistically significant parameter estimate observed for *p*-coumaric acid. The statistical significance of the parameter estimate for quercetin was observed at the intercept, with a *p*-value of less than 0.0001 and a ChiSquare value of 44.84. The parameter estimate for ferulic acid showed a strong statistical significance at the intercept, with a *p*-value of less than 0.0001 and a ChiSquare value of 23.45. One of the most important factors to consider is the maximal specific growth rate (μmax). As demonstrated in Table 7.3, the microorganism was assessed by cultivating it in batch systems and analysing the experimental data collected during the exponential phase of growth.

The target isolates of *N. gonorrhoeae* exhibited significant variation in their growth rates when exposed to different phenolic and flavonoid compounds. This variability indicates the differing impacts of these compounds on bacterial proliferation. The isolates showed a more rapid growth rate when exposed to *p*-coumaric acid, suggesting that this compound may not be effective in inhibiting the growth of *N. gonorrhoeae* and could potentially even promote bacterial proliferation under certain conditions. There was a noticeable decrease in growth activity when the isolates were cultivated in the presence of flavonoid compounds. This aligns with previous research highlighting the antibacterial properties of flavonoids, which are known to inhibit the growth of harmful

bacteria through multiple mechanisms (Roy et al., 2022; Tungmunnithum et al., 2018). Flavonoids function by creating complexes with proteins in the bacterial cell wall, preventing attachment and proliferation, blocking bacterial enzymes like tyrosyl tRNA synthetase, disrupting nucleic acid production, altering membrane function, hindering energy metabolism, preventing cell aggregation and biofilm formation, and reducing pathogenicity. These multifaceted actions underscore the potential of flavonoids as broad-spectrum antibacterial agents. The isolates demonstrated a remarkable ability to adapt to *p*-coumaric acid and ferulic acid, more so than to protocatechuic acid. This adaptability indicates that some phenolic compounds may lose efficacy over time due to bacterial adaptation mechanisms. Ferulic acid did not significantly impact the growth promotion of certain isolates, such as ISID 46, while *p*-coumaric acid had a minimal effect on the growth rate of ISID 54. This differential response suggests that the effectiveness of phenolic compounds can vary significantly among different bacterial strains. *p*-Coumaric acid is believed to induce permanent changes in the bacterial cell membrane, leading to a loss of vital molecules from the cytoplasm and binding to DNA, thereby hindering various cellular functions. This provides insight into the specific antibacterial mechanisms of *p*-coumaric acid. The statistical analysis of protocatechuic acid's action revealed an average value of 7.19, with a standard deviation of 0.25, and a standard error of the mean of 0.08. The 95% confidence interval ranged from 7.01 to 7.37, indicating a moderate level of precision in the observed effects of protocatechuic acid.

Table 7.3: Highest rate of growth observed for the target isolates when exposed to the specific compounds.

	μ_{max} (OD/h)				
ISOLATE ID	**protocatechuic acid**	**ferulic acid**	***p*-coumaric acid**	**quercetin**	**rutin**
ATCC 49226	0.030	0.037	0.043	0.025	0.016
WHO L	0.025	0.042	0.045	0.028	0.009
ISID 5	0.016	0.043	0.169	0.019	0.012
ISID 7	0.042	0.039	0.128	0.029	0.014
ISID 21	0.051	0.034	0.040	0.020	0.013
ISID 26	0.039	0.036	0.069	0.030	0.016
ISID 27	0.039	0.034	0.025	0.018	0.011
ISID 45	0.021	0.047	0.072	0.023	0.012
ISID 46	0.018	0.039	0.114	0.023	0.008
ISID 54	0.036	0.047	0.168	0.033	0.009
ISID 59	0.016	0.044	0.125	0.028	0.007
ISID 60	0.024	0.038	0.033	0.010	0.006

The investigation into the accurate determination of the relationship between optical density (OD) and cellular concentration (cells/mL) was driven by the need for precision in microbiological studies, where accurate cell quantification is paramount. Four different types of bacteria, each with distinct cell sizes and growth characteristics were selected, to ensure that the findings would be broadly applicable. The bacteria were cultivated in a 96-well plate setup, which allowed for high-throughput analysis and consistency in experimental conditions. This setup facilitated the simultaneous measurement of multiple samples, thereby increasing the robustness of the data. Recognizing the importance of accurate OD measurements, the plate reader was meticulously calibrated. Calibration involved using standard solutions of known concentrations to establish a baseline, ensuring the plate reader's light source, detector sensitivity, and wavelength settings were optimal for detecting the bacterial strains used in the study, and performing repeated measurements to confirm the consistency and reliability of the readings. A calibration curve by plotting OD against known concentrations of bacterial cells (cells/mL) was generated. This process involved preparing a series of dilutions from a high-concentration bacterial culture, measuring the OD for each dilution, counting the actual number of cells per millilitre using a hemocytometer or flow cytometry, and plotting the OD readings against the corresponding cell counts to create a standard curve. This curve allowed us to convert OD measurements directly into cell concentrations with high accuracy.

To complement the calibration curve, the growth curves of each bacterial strain by measuring OD at regular intervals during their growth phases (lag, exponential, stationary) was recorded. This data provided insights into the growth dynamics and the relationship between OD and cell density at different stages of growth. By correlating OD measurements with cell counts at various growth phases, it was possible to validate the calibration curve across a range of cellular concentrations. By combining the calibration curve with the growth curve data, the OD correspondence to cellular concentration across different conditions was analysed. This analysis revealed the non-linear nature of the OD-to-cell density relationship at high cell concentrations due to light scattering and absorption limitations, variations in the relationship due to differences in bacterial cell size and morphology, and the influence of the plate setup, such as well size and path length, on OD measurements. The thorough calibration and validation process demonstrated that quantifying cellular density in cells per millilitre (cells/mL) is a more accurate and reliable metric than relying solely on OD measurements. This approach accounted for variations in cell size, growth phase, and experimental conditions, allowing for more consistent and comparable results across different studies. By establishing a reliable method to convert OD to cells/mL, a standardized approach that enhances the reproducibility and comparability of microbiological experiments was provided.

Figure 7.2 showcases the stationary phase of a chromatographic system, where the mobile phase has achieved equilibrium, resulting in the visible representation of the stationary phase. The graph provided depicts the relationship between target isolates and the concentrations of two compounds, *p*-coumaric acid (in blue) and rutin (in red). The data points represent individual measurements for each isolate, and the smooth lines represent trend lines for each compound. The concentration of *p*-coumaric acid follows a non-linear trend across the isolates, initially decreasing in from ISID 5, reaching a minimum around ISID 21, and then increasing again towards ISID 60. The lowest concentration is observed around ISID 21, while the highest concentrations are at the beginning (ISID 5) and the end (ISID 60) of the range. In contrast, the concentration of rutin starts low, increases to a peak around ISID 26, and then decreases again towards ISID 60. The highest concentration is observed around ISID 26, with the lowest concentrations at the beginning (ISID 5) and the end (ISID 60) of the range, similar to *p*-coumaric acid but with different specific values. The graph illustrates the variability in the inhibitory properties of *p*-coumaric acid and rutin across different target bacterial isolates, indicating that the antibacterial activity of these compounds is not uniform across the same bacterial species with deferent resistance profiles, which are influenced by the genetic or metabolic differences among the isolates. For *p*-coumaric acid, the decreasing trend from ISID 5 to ISID 21 followed by an increasing trend to ISID 60 suggests that certain isolates have a significantly lower capacity to produce or accumulate *p*-coumaric acid,

potentially due to different enzymatic activities or metabolic pathways. The initial and final peaks suggest that certain isolates either have enhanced production or better retention of *p*-coumaric acid. For rutin, the increasing trend from ISID 5 to ISID 26 and the subsequent decrease indicates that isolates around ISID 26 have a higher resistance capacity against rutin.

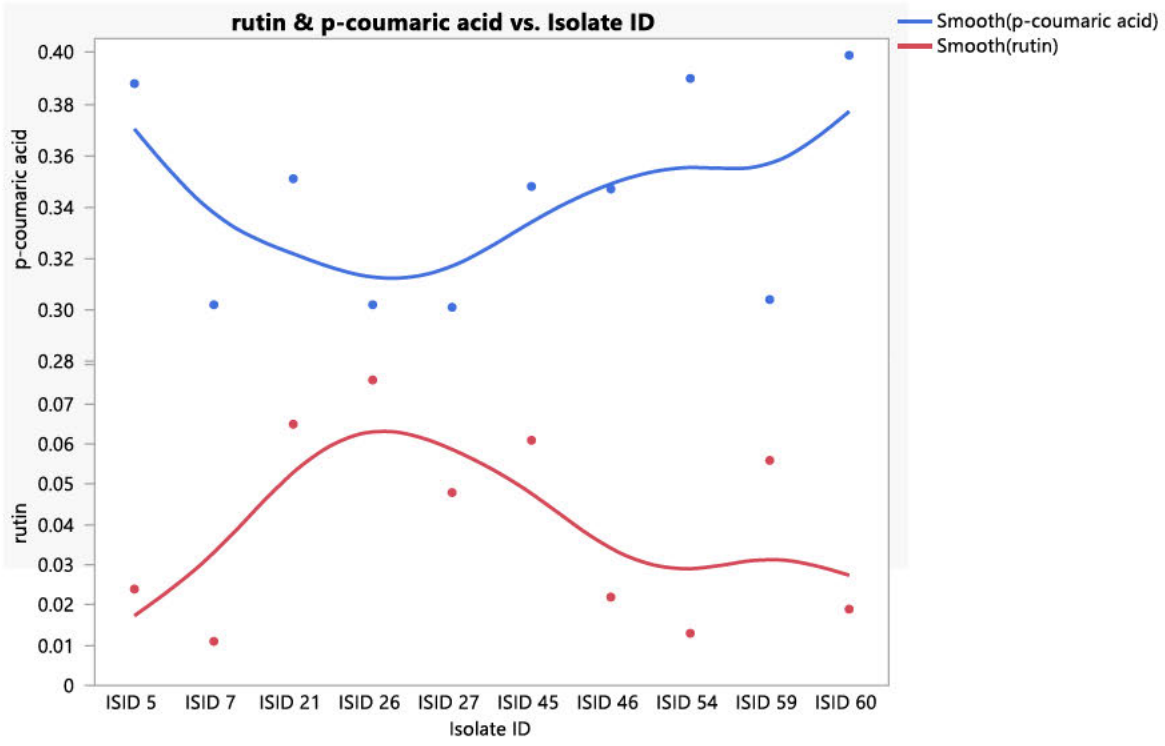

Figure 7.2: The observed optical density per hour (Ymax (OD)) at the stationary phase for the target isolates in the presence of *p*-coumaric acid and rutin.

7.4.3 Effects of combined phenolic compounds on probiotic growth

After ferulic acid mixed with either protocatechuic acid or *p*-coumaric acid, it was seen that the resulting mixture selectively boosted the growth of the isolates, as shown in Figure 7.3. The current body of scientific literature exploring the

effects of phenolic compound combinations exhibiting prebiotic or antimicrobial properties is relatively scarce.

The results of the experiment shown in Figure 7.3 show that mixing phenolic compounds with resveratrol in a stoichiometrically balanced way made them more effective at killing microbes. In the context of combining flavonoids with other compounds, the experimental findings demonstrated notable antibacterial efficacy. Notably, the flavonoids quercetin and rutin exhibited the most optimal outcomes. Rutin, a flavonoid glycoside, and protocatechuic acid, a phenolic compound, exhibit remarkable antimicrobial prowess. Nonetheless, as time elapses, the inhibitory impact of these bioactive agents wanes, leading to an upsurge in bacterial proliferation. The observed synergistic effect between rutin and resveratrol was found to be effective against all isolates, irrespective of their antibiotic resistance profile.

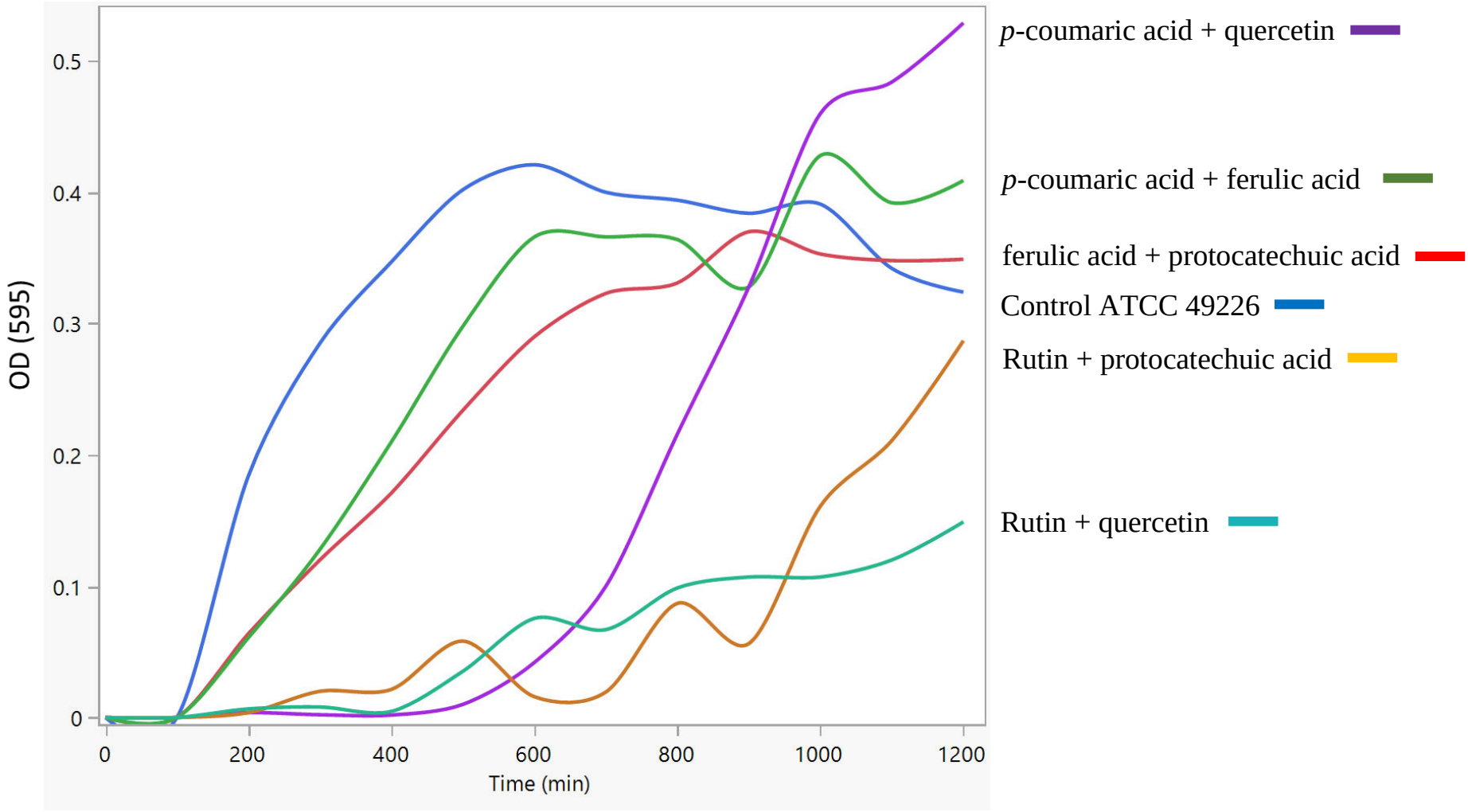

Figure 7.3: Growth curves of dual compounds and negative control (time dependence of OD at wavelength of 595 nm).

The investigation of the antibacterial potential of various phenolic compounds extracted from mushroom sources, specifically targeting clinical isolates of *Neisseria gonorrhoeae*. The compounds tested included phenolic acids (gallic acid, *p*-hydroxybenzoic acid, and protocatechuic acid), cannamic acids (ferulic acid, *o*-coumaric acid, and *p*-coumaric acid), and flavonoids (quercetin and rutin). Among these, quercetin exhibited the most potent antimicrobial activity with a mean MIC of 1.8 µg/mL, followed by rutin with a mean MIC of 3.6 µg/mL. Conversely, *p*-hydroxybenzoic acid and *o*-coumaric acid demonstrated minimal antibacterial activity, with MIC values exceeding 50 µg/mL, suggesting limited effectiveness against the isolates tested. The study's kinetic growth assay revealed that the growth rates of the isolates varied significantly depending on the compound used. *p*-Coumaric acid was noted for its ability to enhance the growth of the isolates, indicating a possible adaptive response by the bacteria. On the other hand, flavonoid compounds, particularly quercetin, inhibited bacterial growth, underscoring their potential as antibacterial agents. The statistical analysis further supported these findings, with quercetin showing a high degree of statistical significance in its antibacterial activity (ChiSquare value of 44.84, $p < 0.0001$). Furthermore, the study evaluated the ADME properties and drug-likeness of the compounds using the SWISSADME tool. Quercetin and ferulic acid demonstrated favourable drug-likeness profiles, with high gastrointestinal absorption and no violations of Lipinski's rule of five, suggesting their potential as therapeutic agents. Rutin, despite its strong antibacterial activity, showed low

bioavailability and poor drug-likeness due to its high molecular weight and multiple hydrogen bond donors and acceptors. The significance of these results lies in their potential application in addressing the growing issue of multidrug-resistant *Neisseria gonorrhoeae*. The potent antibacterial activity of quercetin and ferulic acid, coupled with their favourable pharmacokinetic properties, positions them as promising candidates for further development into therapeutic agents. The study also highlights the complexity of bacterial responses to phenolic compounds, suggesting that while some compounds may inhibit bacterial growth, others may inadvertently promote it. This underscores the need for careful consideration and comprehensive testing when developing new antibacterial treatments. Moreover, the study's insights into the ADME properties of these compounds provide a crucial foundation for future drug development, emphasizing the importance of bioavailability and drug-likeness in the efficacy of potential therapeutics.

7.4 CONCLUSION

The study's findings underscore the intricate dynamics between *N. gonorrhoeae* isolates and various phenolic and flavonoid compounds, highlighting their potential and limitations as antibacterial agents. The significant variation in growth rates among the target isolates when exposed to different compounds reveals the complexity of bacterial responses. Notably, *p*-coumaric acid exhibited limited effectiveness, sometimes even promoting bacterial growth, indicating its

unsuitability as an antibacterial agent for *N. gonorrhoeae*. Conversely, flavonoid compounds demonstrated a marked decrease in bacterial growth activity, aligning with their well-documented antibacterial properties through mechanisms such as protein complex formation, enzyme inhibition, and membrane disruption. The study also highlights the adaptability of bacterial isolates to certain phenolic acids, with ferulic and *p*-coumaric acids showing variable effectiveness across different strains. However, the differential responses to these compounds among target strains emphasize the need for tailored approaches in developing antimicrobial therapies. The overall findings suggest that while certain compounds like quercetin and ferulic acid show strong potential, others like *p*-coumaric acid may not be as effective. This variability highlights the importance of continued research to optimize the use of these compounds against drug-resistant *N. gonorrhoeae*. This study provides valuable insights into the antibacterial capabilities of phenolic and flavonoid compounds, with significant implications for developing novel therapeutic strategies against multidrug-resistant *N. gonorrhoeae* infections. The observed variability in effectiveness underscores the need for further investigation to fully harness the potential of these compounds in clinical applications.

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CHAPTER 8: INVESTIGATION INTO THE INTERACTION BETWEEN PENICILLIN-RESISTANT AND SUSCEPTIBLE GONOCOCCAL PENICILLIN BINDING PROTEIN-2 AND TARGET PHENOLIC LIGANDS THROUGH MOLECULAR DOCKING STUDIES AND STRUCTURE-ACTIVITY RELATIONSHIP ANALYSIS

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Preface

This research focused on two key macromolecules: Penicillin-Binding Protein 2 (PBP2) from *Neisseria gonorrhoeae* strains (penicillin-resistant mutant strain FA6140 (PDB ID: 6HZJ) and the susceptible strain FA19 (PDB ID: 3EQU)), an essential enzyme for cell membrane synthesis. These macromolecules served as the basis for further structural and functional analysis, providing insights the potential as inhibitory interactions using five target phenolic compounds previously identified for their antimicrobial properties in the previous chapter.

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KEYWORDS

Phytochemicals, Penicillin-Binding Protein 2, Ligand-Protein Interaction, Binding Energy, *Neisseria gonorrhoeae* FA19, flavonoids

8.1 ABSTRACT

Gonococcal infections present a notable public health issue, and the major approach for treatment involves using β-lactam antibiotics that specifically target penicillin-binding protein 2 (PBP2) in *Neisseria gonorrhoeae*. This study examines the influence of flavonoids, namely rutin, on the structural changes of PBP2 in both penicillin-resistant (FA6140) and penicillin-susceptible (FA19) strains. The research starts by clarifying the structural effects of certain mutations, such as the insertion of an aspartate residue at position 345 (As*p*-345a), in the PBP2 protein. The strain FA6140, which is resistant to penicillin, shows specific changes that lead to a decrease in penicillin binding. These mutations, namely P551S and F504L, have a significant impact on the pace at which acylation occurs and the stability of the strain under high temperatures. Molecular docking analyses investigate the antibacterial activities of rutin and other phytocompounds, emphasising rutin's exceptional binding affinity and its potential as an inhibitor of PBP2. Quercetin and protocatechuic acid have

encouraging antibacterial effectiveness, with quercetin displaying characteristics similar to those of drugs. Molecular dynamics simulations offer a detailed comprehension of the interactions between flavonoids and PBP2, highlighting rutin's exceptional antioxidant effects and strong affinity for the substrate binding site. The study's wider ramifications pertain to the pressing requirement for antiviral treatments, namely in the context of the ongoing COVID-19 epidemic. Flavonoids have a strong affinity for binding to PBP2, indicating their potential as inhibitors to impair cell wall formation in *N. gonorrhoeae*. Ultimately, this study provides extensive knowledge on the interactions between proteins and ligands, the dynamics of the structure, and the ability of flavonoids to combat penicillin-resistant *N. gonorrhoeae* bacteria. The verified simulation outcomes establish a basis for the creation of potent inhibitors and medicinal therapies to combat infectious illnesses.

8.2INTRODUCTION

Historically, the sexually transmitted illness gonorrhoeae, which is caused by the bacterium *Neisseria gonorrhoeae* (*N. gonorrhoeae*), was effectively treated by delivering a solitary dosage of penicillin (Maduna et al., 2020; Yakobi et al., 2022; Yakobi & Pooe, 2023). However, the emergence of penicillin-resistant bacterial species has led to the exploration of other antibiotics (Allen et al., 2013). The increase in *N. gonorrhoeae* strains exhibiting intermediate resistance to routinely prescribed anti-gonococcal medication poses a significant obstacle to the efficacy of treatment (Gottlieb et al., 2020; Yakobi et al., 2023). Recent research conducted by Vincent and Jerse (2019) has revealed alarming evidence of an escalating trend in overall resistance, which may provide difficulties in selecting appropriate treatment options (Vincent & Jerse, 2019). The antibacterial activities of β-lactam antibiotics are attributed to their specific binding to penicillin-binding proteins (PBPs) (Nakayama et al., 2016). PBPs crucial enzymes responsible for the production of peptidoglycan in bacterial cells, may be classified into three distinct classes (A, B, and C) based on their specific structural and functional characteristics (Niode et al., 2021; Yakobi & Pooe, 2023). Comprehending the transpeptidase function of PBPs in classes A and B is essential for promoting the creation of peptide cross-links between adjacent peptidoglycan strands. Class A PBPs possess a transglycosylase domain, which is accountable for the process of polymerization and the formation of covalent bonds between glycan chains. The study conducted by Straume et al. (2021)

(Straume et al., 2021) emphasises the unique characteristics of Class B PBPs. PBP1, PBP2, and PBPs 3 and 4 are categorised into classes A, B, and C, respectively (Öztürk et al., 2015). PBP2 enzymes have been identified as the principal target of penicillin at the MIC in susceptible bacteria (Kowalska-Krochmal & Dudek-Wicher, 2021; Yakobi et al., 2023a; 2023b). The appearance of penicillin-resistant bacteria, caused by PBP2 variations with an insertion of aspartic acid at the amino acid junction 345–346 (As*p*-345a or D-345a), emphasises the need for alternate treatment methods (Powell et al., 2009). The variations exhibit 4–8 substitutions in close proximity to the protein's C-terminus (Bellini et al., 2019; Fedarovich et al., 2014). the work has revealed the exceptional antibacterial activity of phenolic compounds present in *Pleurotus ostreatus* mushrooms. These compounds have demonstrated significant promise in fighting drug-resistant isolates of *N. gonorrhoeae* (Yakobi et al., 2024; Yakobi et al., 2023). This work highlights the need to discover new bioactive chemicals that can effectively fight against antibiotic-resistant bacteria, thereby tackling the growing problem of antimicrobial resistance. Molecular dynamics simulations enhance the understanding of the interactions between phenolic compounds and PBP2. These simulations offer a comprehensive insight into the intricate molecular dynamics, highlighting the antioxidant effects and strong affinity of the compounds for the substrate binding site. The integration of molecular simulations improves the accuracy and comprehensiveness of the research, enabling a detailed investigation of the complex interactions between proteins

and ligands as well as the structural changes over time. This contributes to the progress of drug development methods. Moreover, this study expands the significance of its findings to include more than just gonococcal infections. Natural compounds' great affinity for target proteins positions them as prospective inhibitors capable of affecting cell wall formation in *N. gonorrhoeae*. This presents a flexible method in the larger context of infectious illnesses. Ultimately, this study provides a fresh viewpoint on the molecular foundations of antibiotic resistance in *N. gonorrhoeae*, presenting bioactive chemicals as promising inhibitors. By utilising the benefits of molecular simulations, the research establishes a strong basis for creating powerful inhibitors and medical treatments. This addresses the urgent issue of infectious illnesses and contributes to the worldwide fight against antibiotic resistance.

8.3MATERIAL AND METHODS

Please refer to chapter 3, subsection 3.8 – 3.11, page 109 – 116 for a detailed material and methods protocol.

8.4RESULTS

8.4.1 Identification of Allowed and Disallowed Regions of Protein Backbone Conformation

We used the Procheck software to evaluate the structural integrity of the modelled FA6140 PBP2. Figure 8.1 illustrates the Ramachandran plot analysis, which displays the statistical distribution of the combinations of the backbone dihedral

angles ϕ and ψ. The Ramachandran plot illustrates the allowable range of conformational possibilities for the Phi/Psi torsion angles of an amino acid, X, located within an ala-X-ala tripeptide, thereby defining the theoretically feasible intervals. The plot's narrative is divided into two separate domains based on the presence or absence of steric hindrances among atoms. The area where steric collisions take place is typically referred to as the disallowed region, while the area without such collisions is commonly referred to as the allowed region.

Through a thorough examination of residue distribution within a protein molecule, valuable insights have been gained regarding its structural and functional characteristics. According to the study results, a large portion of the protein, specifically 91.7%, was found in the most favourable regions, which are A, B, and L. In addition, a smaller percentage of 8.3% was observed in other permitted regions, such as a, b, l, and p. Surprisingly, no residues were found in the permissive regions (~a, ~b, ~l, and ~p) or the impermissible regions. Based on the findings, it is evident that the protein under investigation demonstrates remarkable stability and precise folding. It is distinguished by a condensed core and minimal conformational flexibility. Further investigation is necessary to fully understand the implications of these findings on the protein's biological function. The analysis covered a total of 869 residues. All 715 residues examined were determined to be residues other than glycine and proline. In addition, a comprehensive analysis revealed the presence of 14 terminal amino acid residues,

excluding glycine and proline. The study uncovered the presence of 90 glycine residues, marked by triangles, along with 50 proline residues. The findings mentioned provide valuable insights into the composition of the analysed sample. Identifying outliers in a dataset is a common practice in statistical analysis. Outliers are data points that show a significant deviation from the rest of the data. A widely used approach to identify outliers is by applying a criterion that relies on a threshold of two standard deviations away from the mean. Outliers in graphical data representations are often depicted as black. The small-molecule data is represented by solid and dashed lines, indicating the mean and standard deviation values, respectively. Based on the analysis of the Ramachandran plot, it was found that the core region accounted for a significant 93.0%, with a smaller allowance region of 7.0%. Interestingly, no residues were detected in the generously allowed or disallowed regions. After thoroughly analysing all the Ramachandrans, it was found that only three specific residues were detected out of a total of 823.

In the Chi1-chi2 plots, it is worth noting that a small number of specific residues were observed, totalling seven out of the 451 in question. The side-chain parameters showed significant improvement in 5 cases, with no instances of being located within the interior, and no instances of deterioration. Upon analysing the Residue properties, it was found that there was a maximum deviation of 11.8, a total of 93 unfavourable contacts, 3.6 deviations in bond length and angle, and 4

cis-peptides. The G-factors displayed different values for Dihedral, covalent, and overall measurements. The Planar groups demonstrated a perfect adherence to the established parameters, with no deviations or anomalies observed. This is supported by the absence of any highlighted data points. The alignment of a three-dimensional atomic model with its corresponding one-dimensional amino acid sequence. This was accomplished by classifying the model's structural class according to its precise location and the environment in which it is situated. The results were then compared to established structural benchmarks. Based on the results, it is evident that a considerable number of residues, precisely 88.05%, demonstrate an average 3D-1D score that is equal to or higher than 0.1, see Figure 8.1. According to Figure 8.2, a significant majority of the amino acids in the 3D/1D profile have achieved a score of 0.1 or higher. The Procheck software was used to evaluate the structural integrity of the modelled FA19 PBP2.

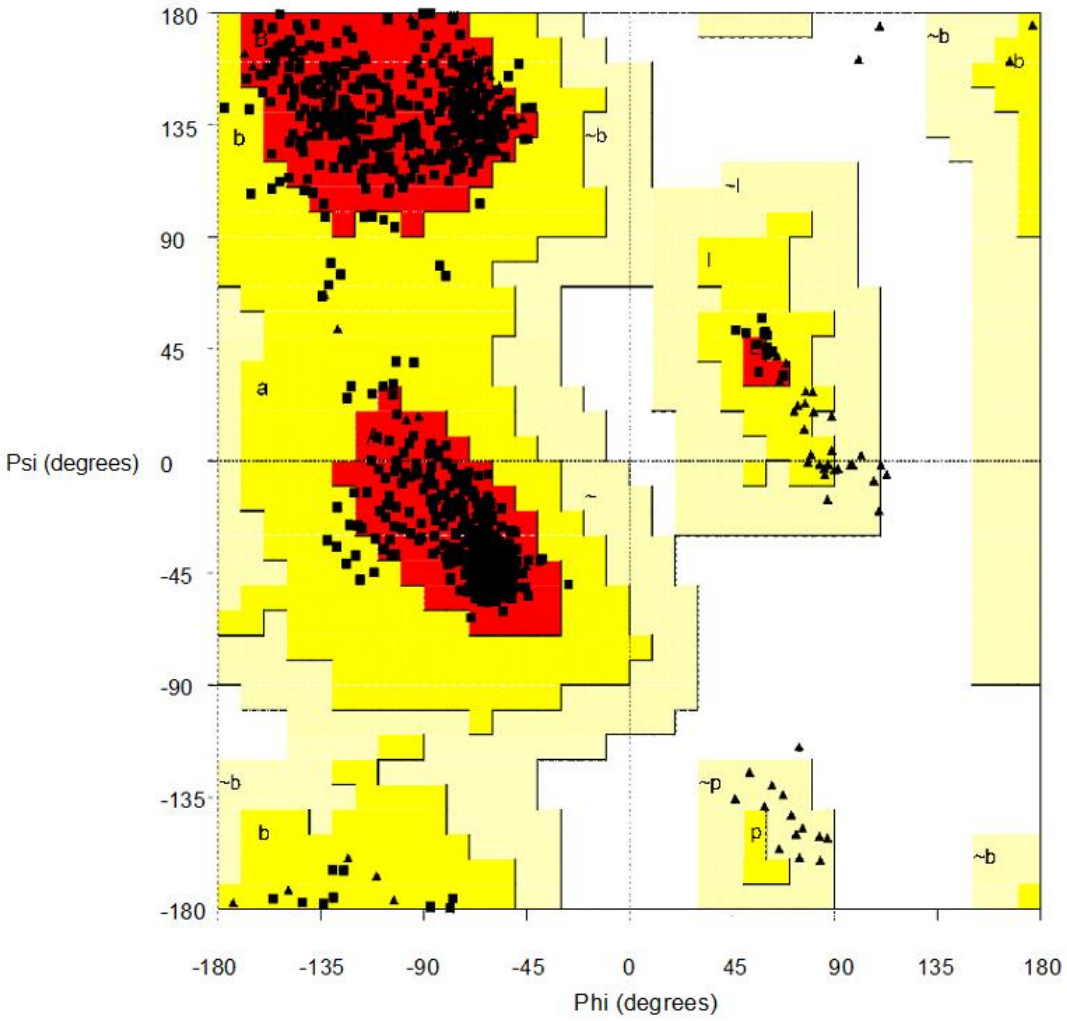

Figure 8.1: The identification of the allowed and disallowed regions of protein backbone conformation was carried out using the Procheck Structure Verification Methodology.

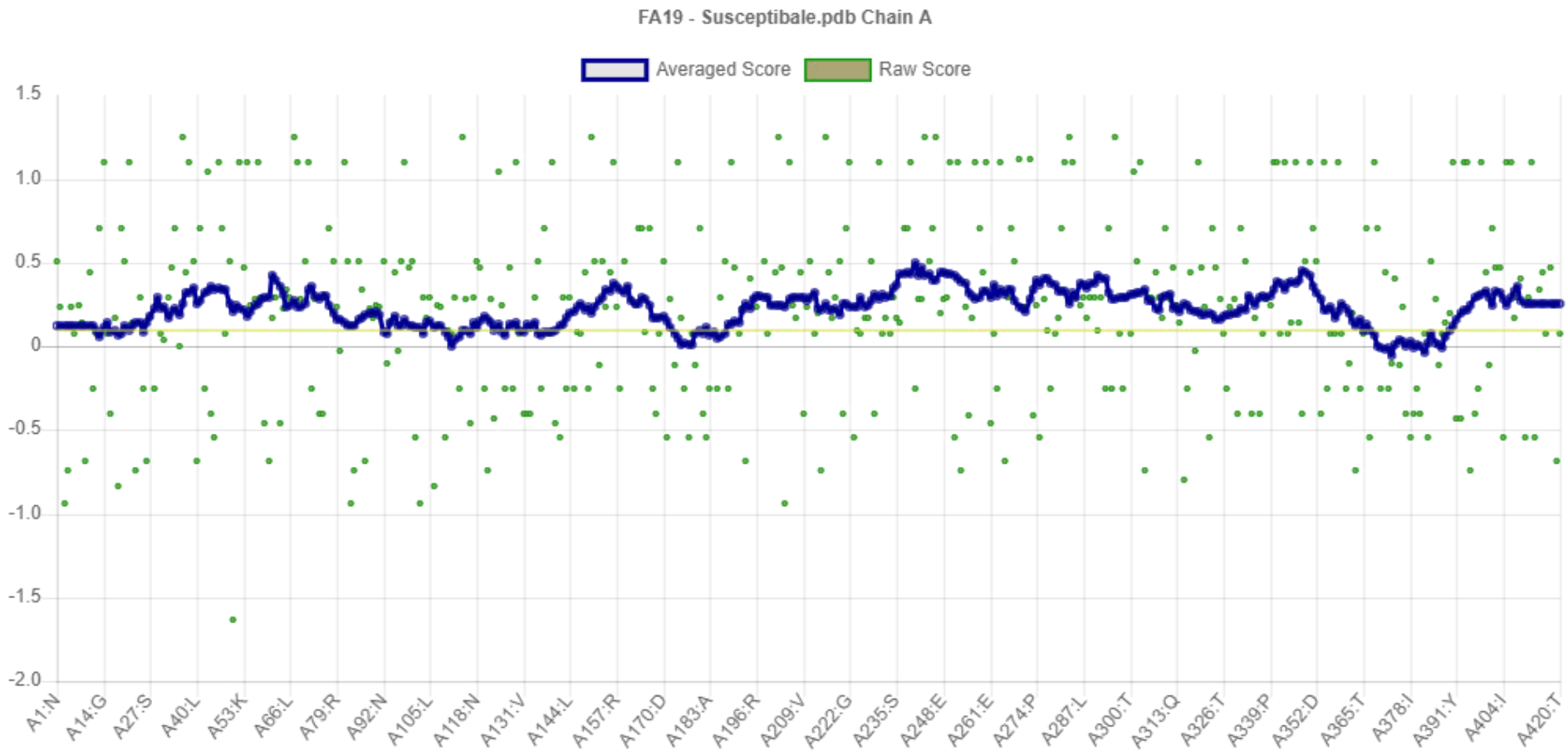

Figure 8.2: The congruence between the three-dimensional (3D) atomic model and the corresponding one-dimensional (1D) amino acid sequence of the FA19 PBP2 chain A was assessed to ensure the accuracy and reliability of the protein structure.

8.4.2 Molecular docking analysis using pyrx and schrödinger

Phytochemicals are natural molecules found in plants that have a crucial function in safeguarding them from both biotic and abiotic threats. Moreover, these compounds possess remarkable bioactive properties that can enhance human health and general well-being. Fruits and vegetables are the primary sources of phenolic compounds in the human diet. Plant-based diets are known to include these main bioactive ingredients, which contribute significantly to their health benefits. The current study utilised PyRx software to conduct molecular docking studies to assess the binding affinity between certain phytochemicals and the FA6140 PBP2 model. The primary objective was to evaluate the binding energy of the mentioned chemicals using computational techniques to replicate the process of binding and scrutinise the ensuing interactions.

The study involved performing molecular docking analyses on five target compounds (see Figure 8.3)—protocatechuic acid, *p*-coumaric acid, ferulic acid, quercetin, and rutin—using the FA6140 PBP2 protein (PDB ID: 6HZJ). The experimental findings indicated that the lowest binding energy was observed in the interaction between the FA6140 PBP2 protein and rutin, followed by quercetin, ferulic acid, *p*-coumaric acid, and protocatechuic acid, respectively. This ranking of binding energies is illustrated in Figure 8.4. The binding energy is significantly influenced by the interactions between ligands and the hydrophobic side chains present in proteins. It is widely recognised that

hydrophobic amino acid residues tend to repel water and other polar functional groups, which can enhance the binding affinity of non-polar compounds. The molecular interaction between rutin and FA6140 PBP2, depicted in Figure 8.5, demonstrates a net attraction of the ligand's non-polar groups to the hydrophobic regions of the protein. This interaction involves the association of non-polar groups or molecules in an aqueous environment.

The results indicate that rutin engages in noncovalent interactions with FA6140 PBP2, involving both hydrogen bonding and hydrophobic interactions. Additionally, the research shows that hydrophobic groups or molecules tend to aggregate in an aqueous medium due to the hydrophobic effect, further stabilising the ligand-protein complex. This phenomenon underscores the importance of considering hydrophobic interactions in molecular docking studies, as they play a crucial role in the binding affinity and stability of the ligand-protein complexes. The molecular docking analysis using PyRx and Schrödinger software revealed that rutin exhibited the highest binding affinity with FA6140 PBP2, followed by quercetin, ferulic acid, *p*-coumaric acid, and protocatechuic acid. The findings highlight the potential of these phytochemicals, especially rutin, as effective inhibitors of PBP2 in *N. gonorrhoeae*, which could lead to new strategies for combating antibiotic resistance.

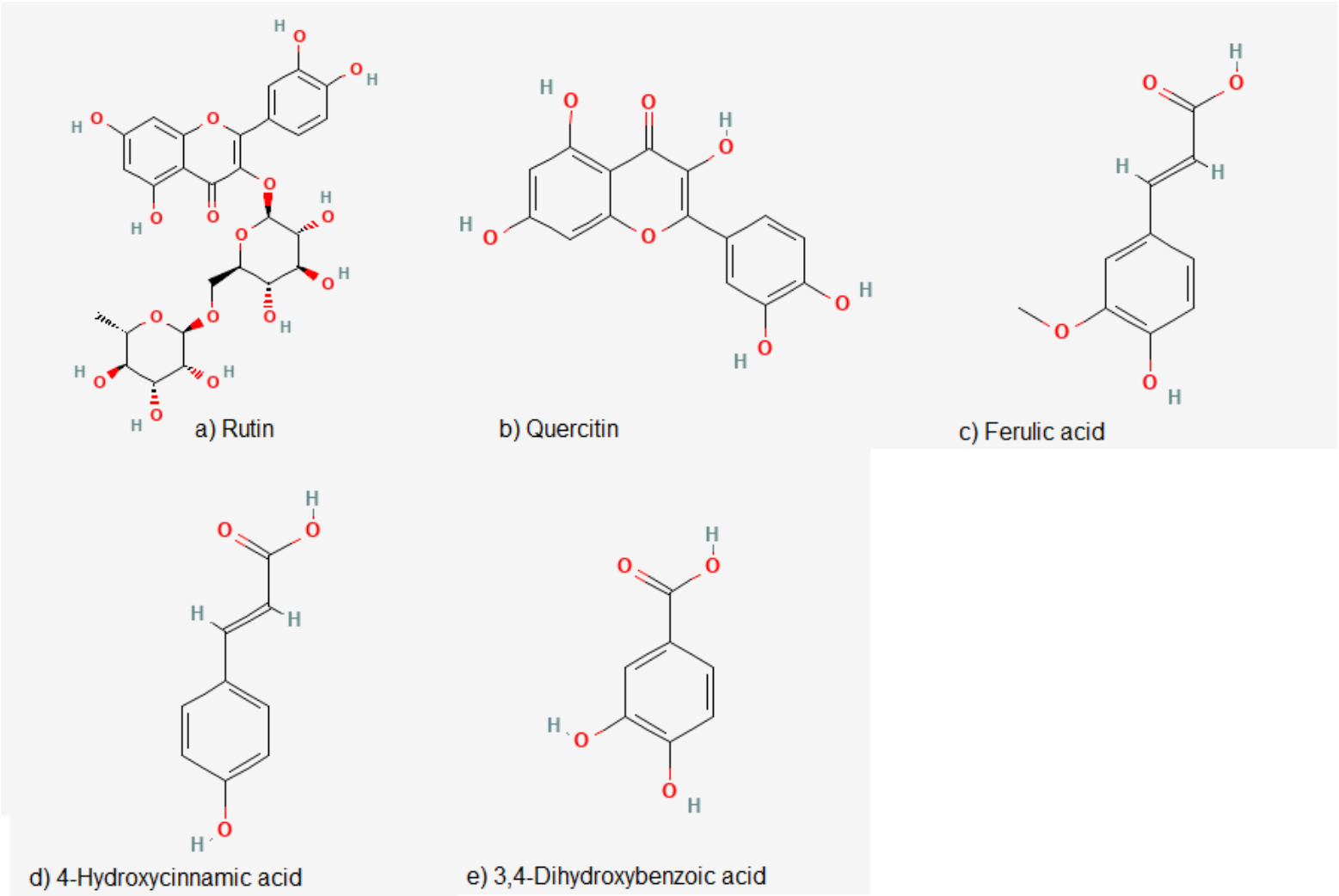

Figure 8.3: Chemical compounds have been identified and thoroughly studied for their ability to combat penicillin-resistant *N. gonorrhoeae* isolates.

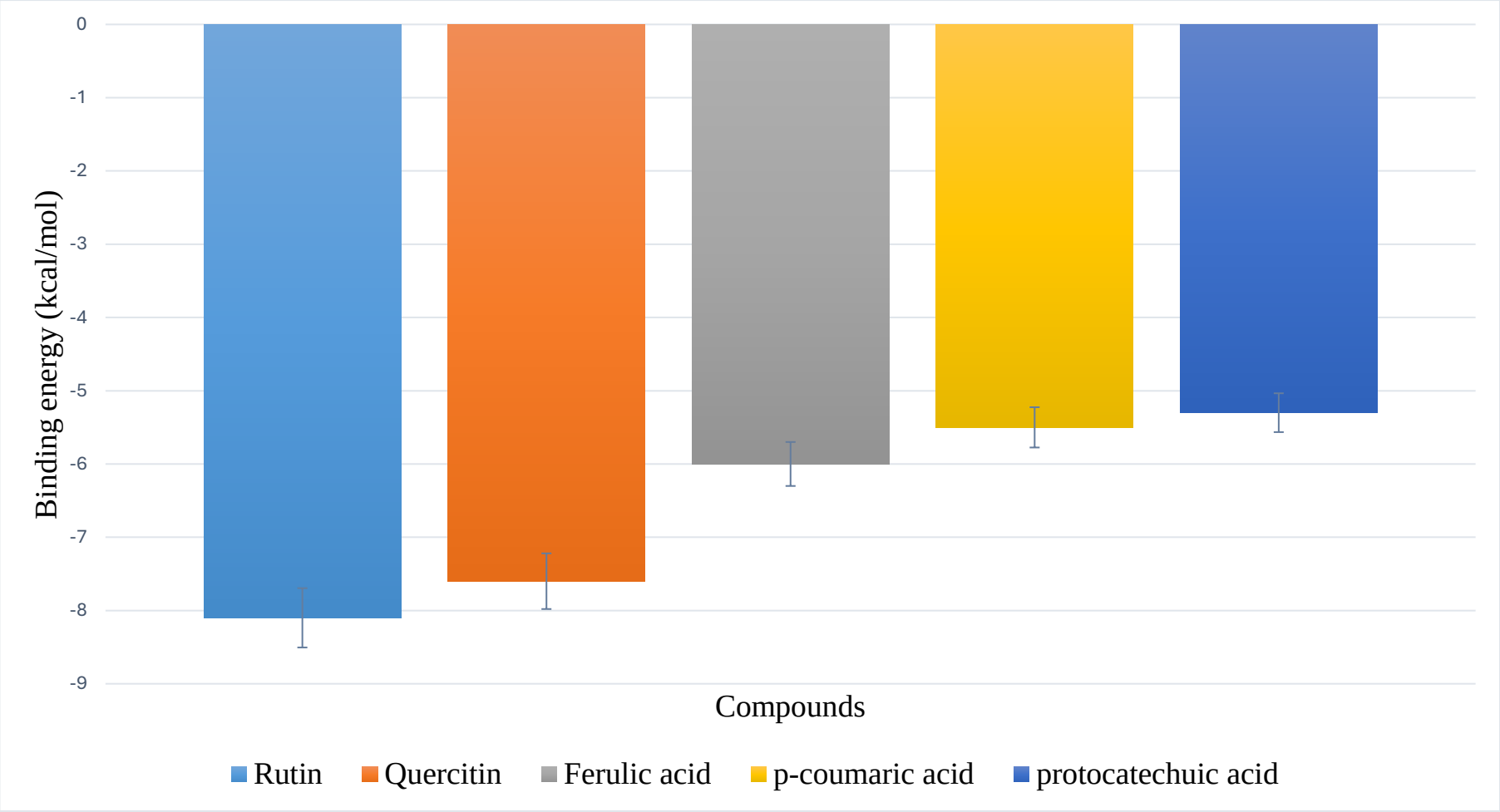

Figure 8.4: Analysis of the binding energy of different compounds.

Phytochemicals, naturally occurring molecules in plants, play a crucial role in protecting plants from both biotic and abiotic stressors. These compounds also possess remarkable bioactive properties beneficial for human health. Fruits and vegetables are primary sources of phenolic compounds, which contribute significantly to the bioactive ingredients in plant-based diets.

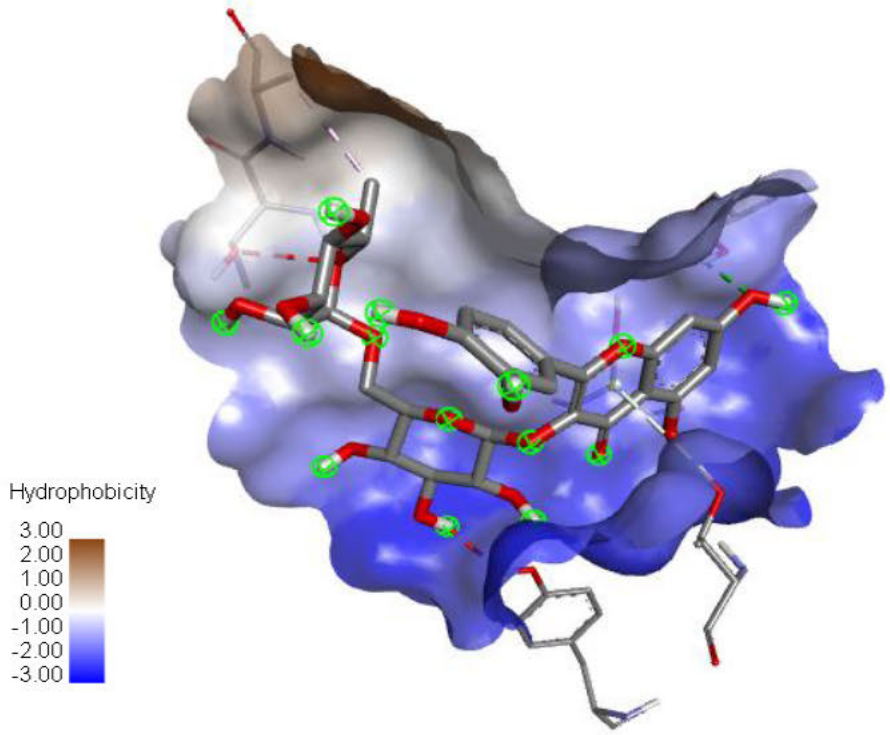

Figure 8.5: The molecular interaction between rutin and FA6140 PBP2, with amino acids such as THR-485, SER-310, THR-347, ALA-485, TYR-350 and SER-362, as well as the behaviour of nonpolar groups or molecules in a water-based setting.

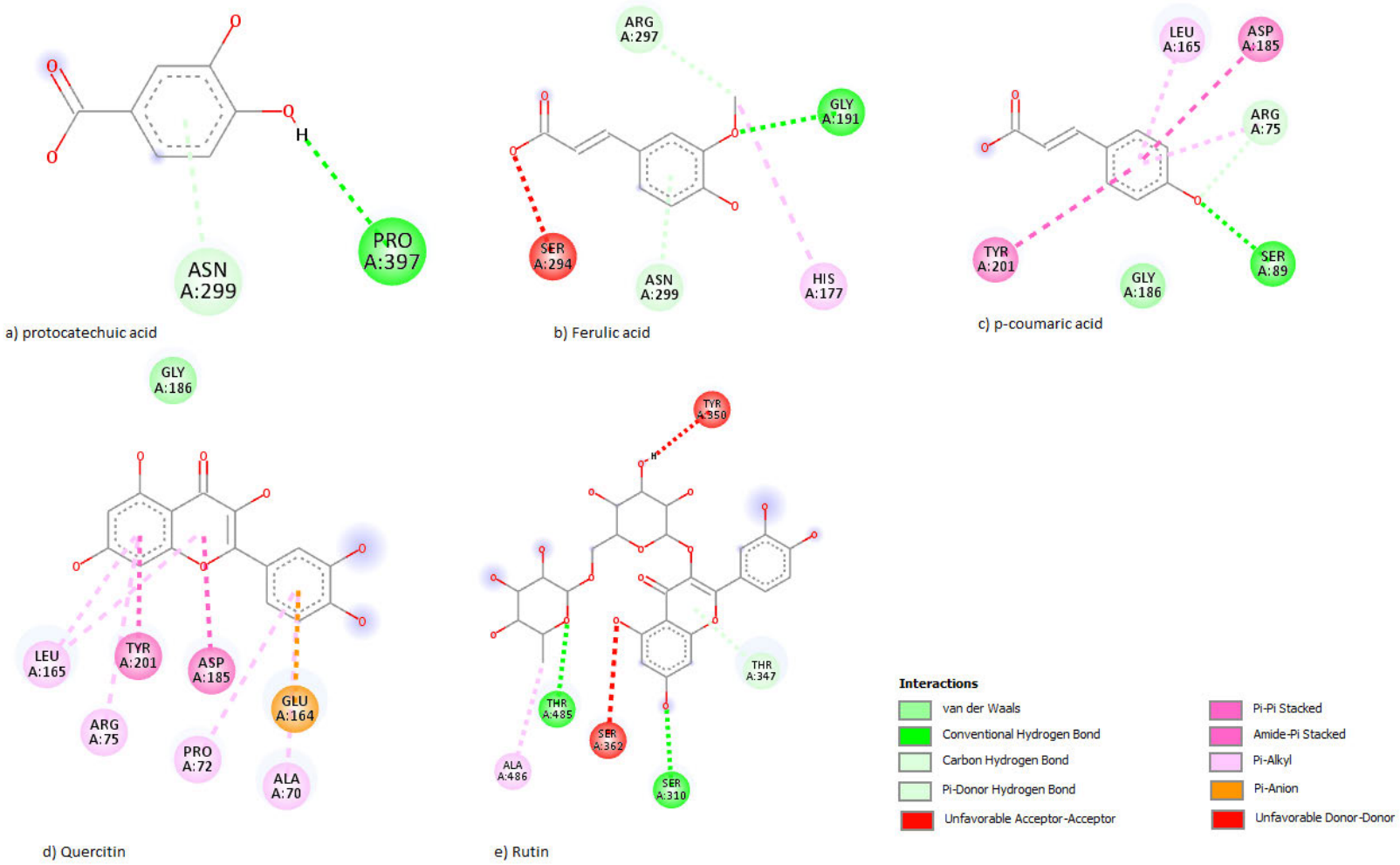

Figure 8.6: Visual depiction of the connections between a specific protein and chosen substances in a two-dimensional format.

In this study, PyRx software was employed to conduct molecular docking studies, assessing the binding affinity between certain phytochemicals and the FA6140 PBP2 model. The goal was to evaluate the binding energies of these compounds using computational techniques to simulate the binding process and analyse the resulting interactions. The docking analyses were performed on five target compounds—protocatechuic acid, *p*-coumaric acid, ferulic acid, quercetin, and rutin—using the FA6140 PBP2 protein (PDB ID: 6HZJ). The findings revealed that rutin had the lowest binding energy, indicating the strongest binding affinity, followed by quercetin, ferulic acid, *p*-coumaric acid, and protocatechuic acid.

8.4.2.1 Charge Distribution in Quercetin-FA6140 PBP2 Interaction

In the context of the interaction between quercetin and FA6140 PBP2, understanding the charge distribution within the molecule or complex system is crucial. The charge distribution can be estimated or calculated to understand the electrostatic interactions between quercetin and FA6140 PBP2. This detailed analysis is essential to grasp the electrostatic contributions to the binding affinity. The interaction between quercetin and FA6140 PBP2 is characterized by robust binding affinity and specific interactions with key amino acids. The docking analysis confirms the molecular basis of the interaction and the conformation of the phytochemicals within the binding sites of FA6140 PBP2. Quercetin exhibited significant binding affinity, forming strong interactions with crucial functional amino acids. These interactions include Pi-Alkyl bonds with LEU-165, ARG-75, PR*O*-72, and ALA-70, van der Waals bonds with GLY-186, Pi-Anion

bonds with GLU-164, and Pi-Pi Stacked and Amine-Pi Stacked bonds with TYR-201 and AS*P*-185, respectively. The thermodynamic stability of the interaction was assessed by computing a docking score of -7.6 kcal/mol. Figure 8.6 illustrates all these bonds together with the interactions and provides evidence of unfavourable donor-acceptor interactions with TYR-350 and SER-362.

8.4.2.2 Interactions of Other Phytochemicals with FA6140 PBP2

Rutin, another compound studied, demonstrated the most significant binding affinity towards FA6140 PBP2, with a docking score of -8.1 kcal/mol. Rutin forms strong hydrogen bonds with essential amino acids such as THR-485, SER-310, and a Pi-donor hydrogen bond with THR-347. It also engages in Alkyl interaction with ALA-485 and shows unfavourable donor-donor and acceptor-acceptor interactions with TYR-350 and SER-362, respectively (Figure 8.7). Ferulic acid interacted with PR*O*-191 and ASN-299 through hydrogen bonds and formed a Pi-Alkyl bond with HIS-177. An unfavourable acceptor-acceptor bond was observed with SER-294, and a Pi-donor hydrogen bond was formed with ARG-297. The binding affinity for ferulic acid was calculated to be -6.0 kcal/mol. *p*-Coumaric acid exhibited a binding affinity of -5.5 kcal/mol, engaging in hydrogen bonding interactions with SER-89 and ARG-75, a Pi-Alkyl bond with LEU-165, an Amine-Pi Stacked bond with AS*P*-185, a van der Waals bond with GLY-186, and a Pi-Pi Stacked bond with TYR-201. Lastly, protocatechuic acid formed conventional hydrogen bonds with PR*O*-397 and Pi-donor hydrogen bonds with ASN-299, exhibiting a binding affinity of -5.3 kcal/mol. The

molecular docking analysis using PyRx and Schrödinger revealed that rutin exhibited the highest binding affinity with FA6140 PBP2, followed by quercetin, ferulic acid, *p*-coumaric acid, and protocatechuic acid. These findings highlight the potential of these phytochemicals, especially rutin and quercetin, as effective inhibitors of PBP2 in *N. gonorrhoeae*. This could lead to new strategies for combating antibiotic resistance in this pathogen.

The results obtained from the docking analysis confirm the molecular basis of how the designated phytochemicals interact and fit into the binding sites of FA19 PBP2. The results of the docking study are presented in Table 8.1, which aimed to predict the binding strength and arrangement of the compounds in the active site of the protein. In addition, the research indicates that the phytochemicals have the capacity to create numerous hydrogen bonds and non-covalent interactions with the crucial functional residues of the protein under investigation. Based on extensive research in the field, it has been found that rutin demonstrates a significantly stronger binding affinity to the target protein when compared to other compounds. The molecular entity shows a strong ability to form hydrogen bonds with important amino acid residues, leading to a docking score of -8.1 kcal/mol. There was an observed interaction between rutin and the amino acid THR-347, which falls under the category of a carbon-hydrogen bond. In addition, it forms an Alkyl linkage with ALA-496, as shown in Table 8.1. In addition, the compound quercetin showed strong binding to the specific protein and formed

strong interactions with important amino acids involved in its function. These interactions included hydrogen bonds at LEU-165, SER-89, and TYR-201, van der Waals bonds at GLY-186, Pi-Anion bonds at GLU-164, Pi-Pi Stacked bonds and Amine-Pi Stacked bonds at TYR-201 and AS*P*-185, respectively, and Pi-Alkyl bonds with ARG-75. The thermodynamic stability of the interaction was assessed by calculating the docking score, which resulted in a value of -7.8 kcal/mol, as shown in Table 8.1.

In this study, Schrödinger software was employed to conduct molecular docking analyses, complementing and expanding upon the findings obtained using PyRx. The target protein, 3EQU [A], representing PBP2 from *Neisseria gonorrhoeae* strain FA19, was investigated for its interactions with protocatechuic acid, *p*-coumaric acid, ferulic acid, quercetin, and rutin. The objective was to assess the binding affinities and interaction patterns of these compounds, shedding light on their potential therapeutic roles. The results revealed distinctive docking scores and interaction profiles for each compound.

Protocatechuic acid demonstrated a docking score of -6.1 kcal/mol, forming stable hydrogen bonds with residues LEU-165, AS*P*-204, ALA-73, and TYR-201, along with other interactions involving TYR-75 and ARG-75. Similarly, *p*-coumaric acid exhibited a moderate binding affinity with a docking score of -5.6 kcal/mol, engaging in hydrogen bond interactions with SER-89 and ARG-75, alongside other interactions with LEU-165, AS*P*-185, GLY-186, and TYR-201.

Table 8.1: Molecular docking evaluation of the interactions between various phytochemicals and the 3equ [A] protein, representing Penicillin-Binding Protein 2 (PBP2) from *Neisseria gonorrhoeae* strain FA19.

Compound Name	**PubChem CID**	**Docking Score kcal/mol**	**Hydrogen bond interaction**	**Other interactions**
Protocatechuic acid	72	-6.4	LEU-165, AS*P*-204, ALA-73, TYR-201	TYR-75, ARG-75
p-coumaric acid	637542	-5.7	SER – 89, ARG – 75	LEU – 165 , ASP – 185, GLY – 186, TYR – 201
Ferulic acid	445858	-5.9	TY*P*-201	ARG-75, LEU-165, AGR167
Quercetin	5280343	-7.8	LEU-165, SER-89, TYR-201	GLY-186; GLU-164; TYR-201, AS*P*-185, ARG-75
Rutin	5280805	-8.1	THR-485, SER-310, GLY-481, GLY-482	THR-347, ALA-496

Ferulic acid demonstrated a strong binding affinity with a score of -5.8 kcal/mol, primarily interacting via hydrogen bonds with TYR-201, complemented by interactions with ARG-75, LEU-165, and ARG-167. Quercetin displayed a high binding affinity with a docking score of -7.7 kcal/mol, forming significant hydrogen bonds with LEU-165, SER-89, and TYR-201, as well as other interactions involving GLY-186, GLU-164, TYR-201, AS*P*-185, and ARG-75. Rutin exhibited the highest binding affinity among the compounds, with a docking score of -8.0 kcal/mol, engaging in extensive hydrogen bonding with THR-485, SER-310, GLY-481, and GLY-482, along with other interactions with THR-347 and ALA-496.

The Schrödinger docking analyses corroborated the findings from PyRx, highlighting rutin and quercetin as promising candidates for PBP2 inhibition due to their strong binding affinities and interactions with key residues in the active site. These results emphasize the potential of these phytochemicals in combating multidrug-resistant *N. gonorrhoeae*, warranting further exploration into their therapeutic applications.

8.4.3 Molecular dynamics simulations analysis

After careful evaluation, rutin emerged as the compound with the strongest binding affinity among all the tested compounds. Therefore, it was selected for in-depth analysis using molecular dynamics simulation. The decision was made due to the strong interaction observed between the compound and the target

protein, Penicillin-Binding Protein 2 (PBP2) from *Neisseria gonorrhoeae* strain FA19. This interaction was supported by a high docking score and the formation of multiple hydrogen bonds with important residues in the active site. Using molecular dynamics simulation, a comprehensive understanding of the interaction between rutin and PBP2 can be gain. This approach allows us to observe the stability, conformational changes, and dynamics of the protein-ligand complex as it evolves over time. Through the analysis of the complex under simulated environmental conditions, valuable insights into the intricate molecular mechanisms that govern their interaction can gain. Using molecular dynamics simulation, the goal is to understand how rutin behaves within the binding pocket of PBP2. The interactions with nearby residues and evaluate the stability of the protein-ligand complex in real-life conditions were examined. This thorough analysis provided additional support for the effectiveness of rutin as a potential treatment for multidrug-resistant *N. gonorrhoeae*. It shows promise in the development of a new treatment option for gonorrhoeae.

In addition, the molecular structure of rutin includes several functional groups, such as hydroxyl (-OH) and carbonyl (C=O) groups. These groups facilitate the formation of hydrogen bonds and other non-covalent interactions with specific proteins. These interactions play a crucial role in stabilising the rutin-protein complex and have a significant impact on its binding affinity and specificity. In addition, rutin has been found to have significant pharmacological effects,

including the ability to inhibit enzymes and receptors involved in disease processes. The potential therapeutic applications of this field of study are vast, ranging from cardiovascular disorders and diabetes to cancer and microbial infections. This makes it a highly promising area for further investigation and research. Rutin has demonstrated a high level of bioavailability and a low level of toxicity, making it a suitable option for pharmacological interventions. The pharmacokinetic properties of rutin indicate that it has the potential to easily enter cells and interact with targets inside, leading to its therapeutic effects. In general, the distinctive chemical composition, medicinal characteristics, and advantageous pharmacokinetics of rutin make it a highly suitable candidate for conducting molecular dynamics simulations. These simulations provide valuable information about how rutin interacts with specific proteins and its potential as a therapeutic treatment for a range of illnesses, including drug-resistant bacteria such as *Neisseria gonorrhoeae*.

8.4.3.1 Rutin-penicillin-resistant PBP2

A comprehensive study was carried out on rutin-penicillin-resistant PBP2, involving a detailed analysis of molecular dynamics (MD) simulations using the Desmond package. The data presents the outcomes of Root Mean Square Fluctuation (RMSF) calculations for different atoms in a molecular system. These calculations are commonly used in molecular dynamics simulations to evaluate the flexibility or mobility of atoms in a protein or ligand over a specific time frame. The data contains the index or identifier of the atom within the system, the

name of the residue in the Protein Data Bank (PDB) format, and the RMSF value in relation to the protein. The statement discusses the depiction of the protein's atom fluctuation or flexibility, along with the root mean square fluctuation (RMSF) value in relation to the ligand. The "wrt_Protein" values reflect the diversity of individual atoms within the protein structure. Higher values indicate increased flexibility or mobility of the atoms in question. The "wrt_Ligand" values indicate the level of variation among the atoms in the ligand. Similarly, higher values suggest increased flexibility or mobility of the atoms in the ligand. When Atom# 5 is analysed, it was observed that it had a "wrt_protein" value of 13.084 and a "wrt_ligand" value of 1.527. Based on the results, it appears that one of the atoms in the protein shows some flexibility, and there is also a moderate amount of variability in the ligand. In general, the RMSF values provide valuable insights into the atomic movements within both the protein and the ligand. This information is crucial for understanding the stability and interactions within the molecular system, as depicted in Figure 8.7.

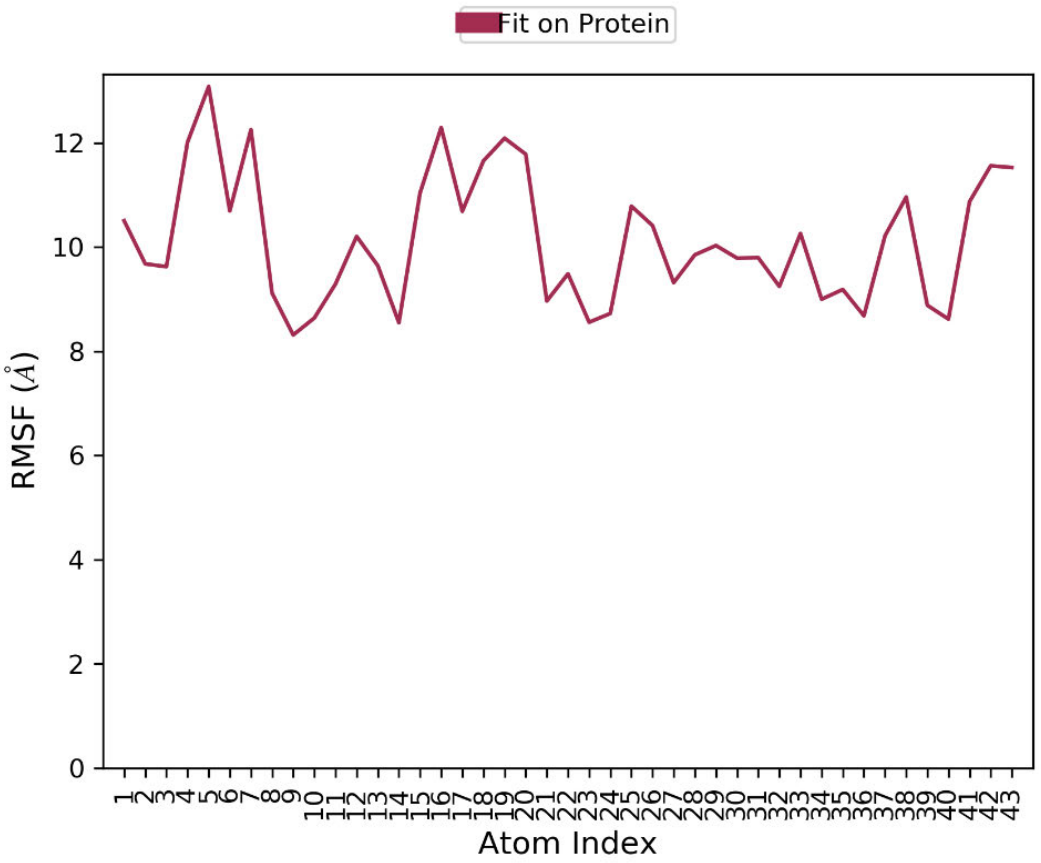

Figure 8.7: The root-mean-square fluctuation (RMSF) analysis of molecular dynamics (MD) simulation provides valuable insights into the dynamic behavior of both the protein and ligand throughout the simulation trajectory.

The torsion angles, also referred to as dihedral angles, in a molecular system are determined by the atomIDs in the system and can provide valuable insights into the molecular structure. These angles measure the rotation between the planes of four consecutive atoms in the molecular structure. The dihedral angle is determined by four atomIDs: ai, aj, ak, and al. Each row in the data represents a unique frame or time point in a molecular dynamics simulation. The columns show the numerical values of the dihedral angles, measured in degrees, for each

specified torsion in the system. The values in each cell of the table indicate the exact angle (measured in degrees) for the corresponding dihedral in a specific frame. Examining a specific entry (entry at Frame 0, dihed1): At Frame 0, the dihedral angle between atoms with the IDs 27, 24, 10, and 64 measures 24.876 degrees. The dihedral angles display a range of variation across successive frames, indicating the dynamic nature of the molecular system, as illustrated in Figure 8.8. The system explores different torsional conformations, as evidenced by the changing values of the dihedral angles. The direction of rotation at these angles can be determined by the presence of positive or negative values. The graphic displaying the ligand torsions offers a clear and concise depiction of the conformational changes in each rotatable bond (RB) within the ligand throughout the entire simulation track, spanning from 0.00 to 100.00 nsec. The upper panel presents a two-dimensional diagram of a ligand, showcasing rotatable bonds that are distinguished by various colours. For each rotatable bond torsion, a dial plot and bar plots that share the same colour was found. Graphs that display the torsion angle throughout the simulation show the configuration in a dial or radial format. The simulation begins at the centre of the radial plot, with the temporal progression illustrated in an outward radial fashion. The bar plots offer a succinct way to showcase the information derived from the dial plots, effectively illustrating the probability density of the torsion. When data on torsional potential was available, the figure showed the potential of the rotatable bond by combining the potential of the corresponding torsions. The y-axis of the chart shows the

potential values, which are positioned to the left and represented in units of kcal/mol. Studying the relationship between the histogram and torsion potential could yield valuable insights into the structural stress that the ligand undergoes to maintain its shape when bound to a protein.

The L_Properties are obtained through a molecular dynamics simulation or a similar computational research method. They capture specific characteristics of a molecular system at various frames or time steps. The simulation's frame or time step, which measures the average deviation of atomic locations between the current frame and a reference structure, offers valuable information about the magnitude of structural changes taking place during the simulation. Understanding the molecular structure's compactness can be measured by the radius of gyration, while the presence of intramolecular hydrogen bonds indicates the quantity of hydrogen bonds formed within the molecule. Understanding the molecular surface area is crucial in grasping the molecule's overall surface characteristics. On the other hand, the solvent accessible surface area focuses on the specific portion of the molecule's surface that can be accessed by solvent molecules. Conversely, the polar surface area pertains to the portion of the molecule's surface that is taken up by polar atoms.

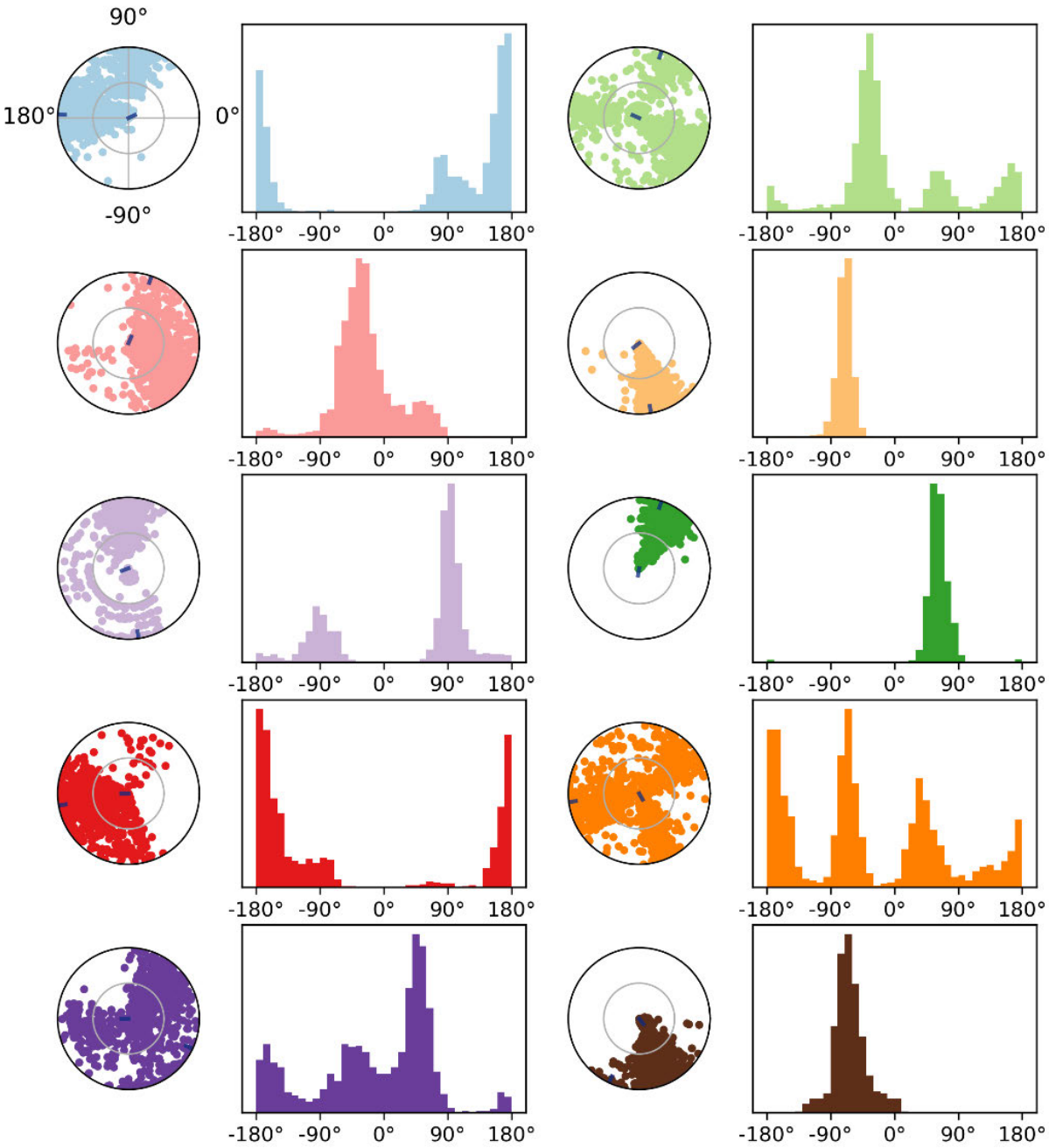

Figure 8.8: Torsion angle profiles play a crucial role in molecular dynamics (MD) simulations as they provide detailed information about the conformational changes and flexibility of molecules over time.

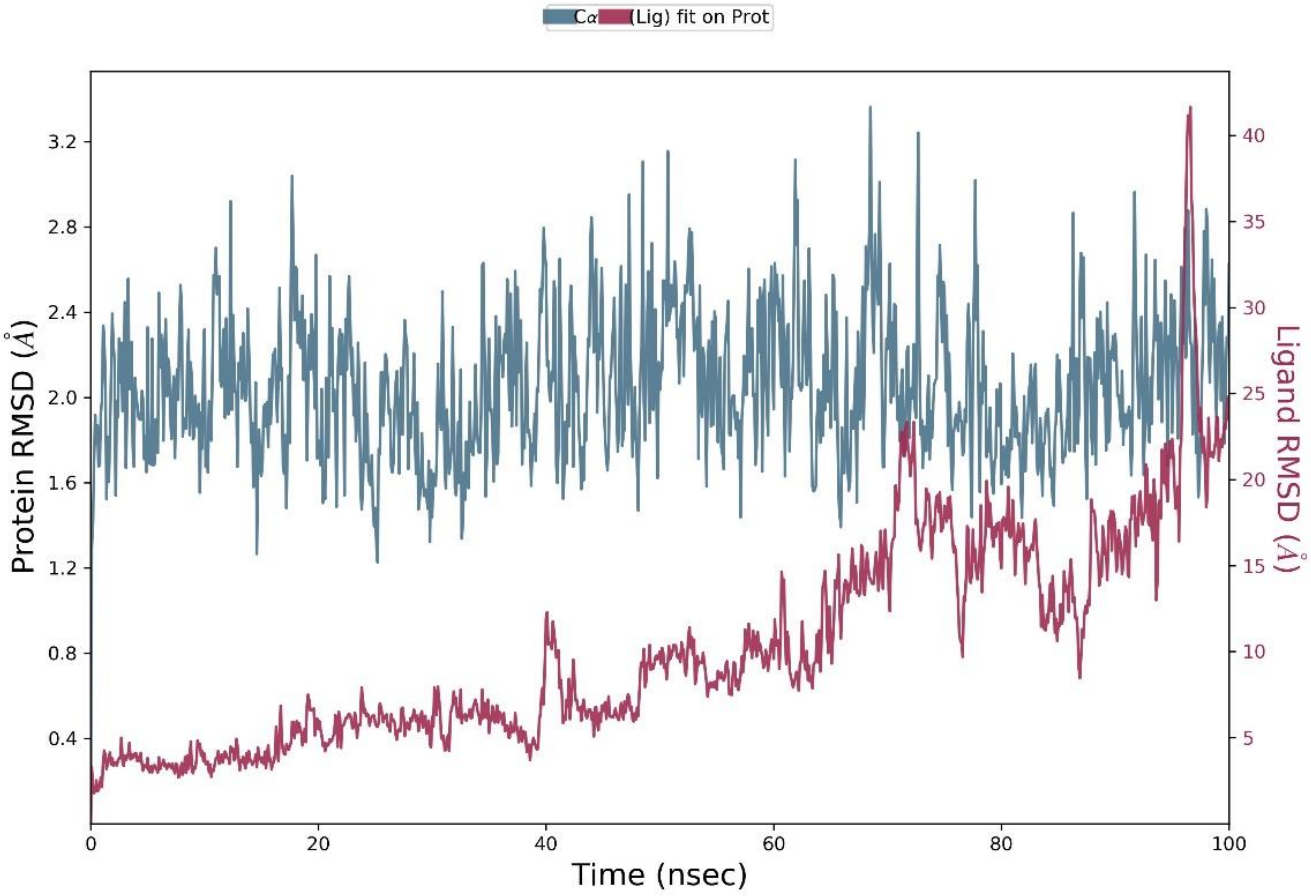

Figure 8.9: Protein-ligand complex investigating the dynamic structural changes of the rutin-penicillin resistant PBP2 complex over time.

The simulation findings indicate that the system undergoes structural changes, as evidenced by an increase in RMSD and fluctuations in other properties. Significantly, there has been a significant rise in RMSD, indicating a more noticeable deviation from the original structure. Certain frames demonstrate a stabilising pattern, with a consistently low root mean square deviation (RMSD) and other consistent properties. On the other hand, some frames exhibit oscillations within the system, but the properties do not exhibit any significant patterns. These interpretations are based on broad patterns, and a more thorough analysis may be needed to draw specific conclusions about the behaviour of the molecular system during the simulation.

The figure shown above (Figure 8.9) demonstrates the gradual alterations in the root mean square deviation (RMSD) of a protein, as indicated on the left y-axis. The protein frames are aligned with the reference frame backbone, and the root-mean-square deviation (RMSD) is computed using the selected atoms. Monitoring the root mean square deviation (RMSD) of the protein can offer valuable insights into its structural conformation throughout the simulation. An analysis of the RMSD can help determine if the simulation has reached equilibrium. This involves examining the fluctuations towards the end of the simulation, which should be similar to the average thermal structure. Small, globular proteins can withstand slight changes in the range of 1-3 Å without experiencing any problems. Notable changes beyond the mentioned extent

indicate that the protein is undergoing a significant structural change during the simulation. In order to ensure the accuracy of your simulation, it is essential to achieve convergence, which occurs when the root mean square deviation (RMSD) values stabilise and remain constant. If the protein's root mean square deviation (RMSD) consistently increases or decreases throughout the simulation, it suggests that the system has not yet achieved equilibrium. Therefore, the length of the simulation may not allow for thorough analysis. The ligand RMSD, displayed on the right y-axis, measures the stability of the ligand in relation to the protein and its binding pocket. The graphic above illustrates the RMSD (root mean square deviation) of a ligand in the protein-ligand complex. The alignment is achieved by matching the protein backbone of the complex to a reference structure, followed by the calculation of the RMSD for the heavy atoms of the ligand. When the measured values significantly exceed the root mean square deviation (RMSD) of the protein, it suggests that the ligand may have moved away from its initial binding site.

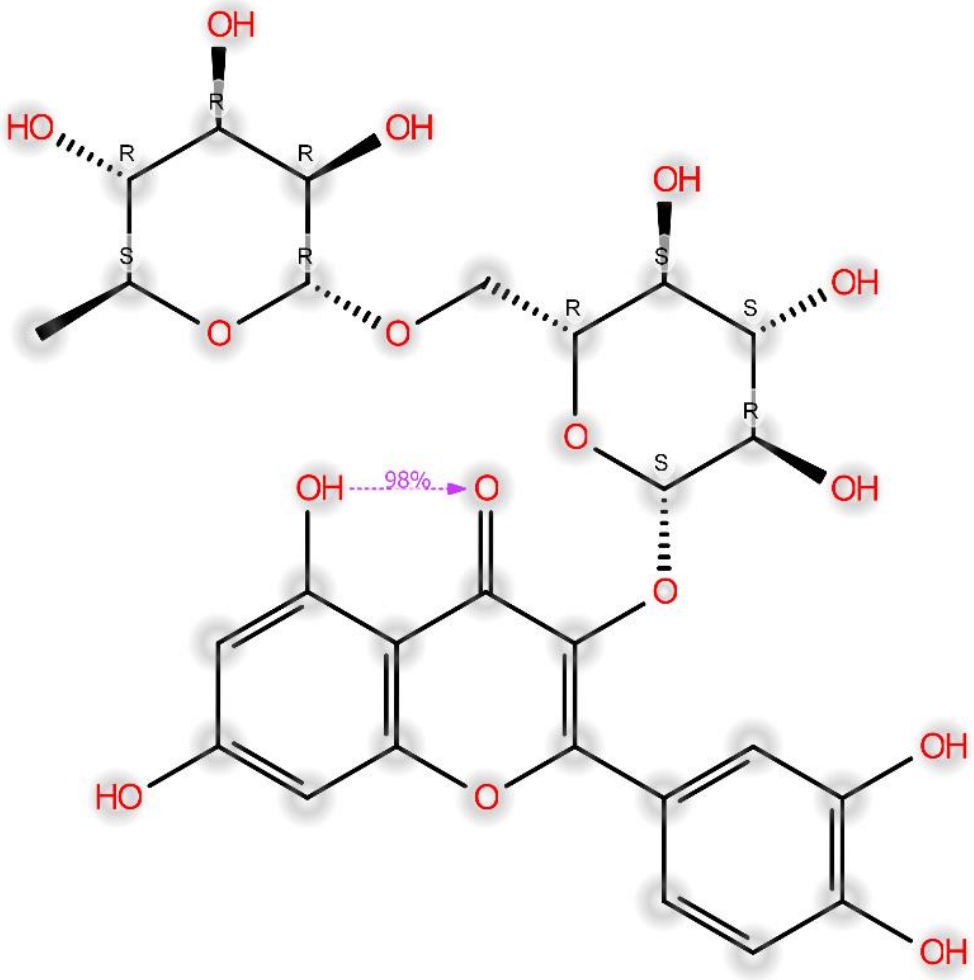

Figure 8.10: Contact analysis between rutin and Penicillin-Binding Protein 2 (PBP2) in *Neisseria gonorrhoeae* provides valuable insights into the specific interactions governing their binding.

Figure 8.10 showcases the precise interactions that occur between the atoms of a ligand and the residues of a protein. Interactions that occur for over 30.0% of the simulation duration in the selected trajectory (0.00 through 100.00 nsec) are shown. It is possible for interactions to exceed 100% in cases where specific residues form multiple interactions with a single ligand atom. For instance, the Arg side chain has four H-bond donors that can potentially create hydrogen bonds

with a single H-bond acceptor. Throughout the simulation, the consistent observation and categorization of the ligand's interactions with proteins were evident in the findings. The classification includes various types of interactions, such as hydrogen bonds, hydrophobic interactions, ionic interactions, and water bridges. Every one of these types of interactions has more specific subtypes, providing a detailed understanding of the characteristics of the interactions. The graph shown in Figure 8.11 offers a succinct overview of the protein-ligand interactions throughout the simulation trajectory. The interactions are carefully standardised, allowing for a precise comparison of their rates. In this case, a value of 0.7 would indicate that the contact in question was maintained for 70% of the experiment's duration. It's important to mention that values greater than 1.0 can occur, indicating that certain protein residues may have multiple interactions of the same kind with the ligand. Through this in-depth analysis, researchers gain a deep understanding of the frequency and consistency of different interactions between the protein and ligand during the simulation. This valuable information provides crucial insights into the dynamics of the molecular complex.

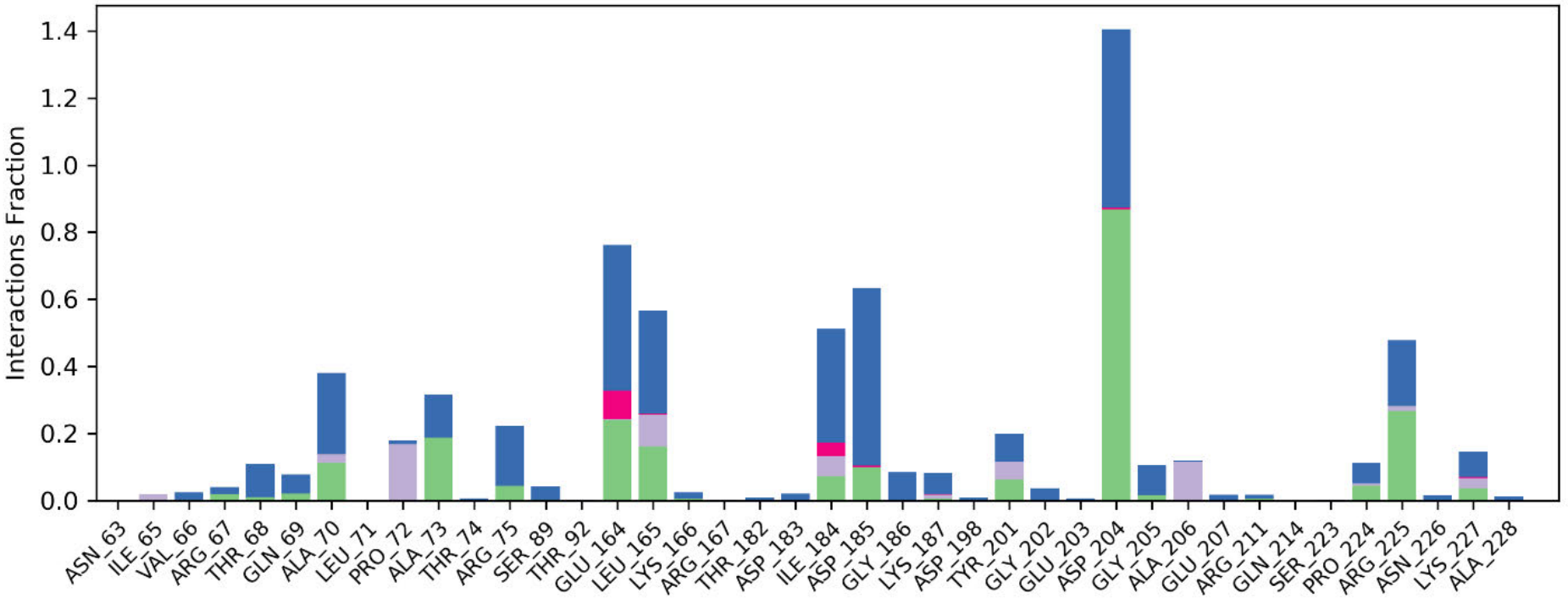

Figure 8.11: Analysing the protein-ligand interaction dynamics involves categorizing contacts over the trajectory of molecular dynamics simulations to understand how the binding between rutin and Penicillin-Binding Protein 2 (PBP2) in *Neisseria gonorrhoeae* evolves over time.

8.4.3.2 Rutin-penicillin-susceptible pbp2

A thorough examination of molecular dynamics (MD) simulations was carried out using the Desmond package to study rutin-penicillin-susceptible PBP2. Atoms 1 to 5 exhibit a noticeable upward trend in the values of "wrt_Protein," indicating a consistent pattern in the measured characteristic in relation to the protein. Atoms 6 to 15 exhibit varying values for both "wrt_Protein" and "wrt_Ligand," suggesting potential differences in how these atoms interact with both the protein and ligand. The values of atoms 16 to 43 exhibit a range of combinations, showing variations in relation to both the protein and the ligand. The Root Mean Square Fluctuation (RMSF) is an important tool in assessing localised variations along the protein chain.

The graphic in Figure 8.12 illustrates the dynamic behaviour of the protein throughout the experiment, with peaks indicating the regions of highest volatility. In general, the N- and C-terminal tails exhibit more pronounced variations in comparison to other regions. On the other hand, secondary structural components like alpha helices and beta strands are known for their increased rigidity compared to unstructured regions. This rigidity results in reduced fluctuation, especially when compared to loop regions. The Ligand Contacts section provides valuable information about the protein residues that interact with the ligand. The interactions are visually represented by vertical bars that are coloured green. Having a deep understanding of the specific residues involved in ligand

interactions greatly improves the overall comprehension of the dynamics of protein-ligand binding during the simulation.

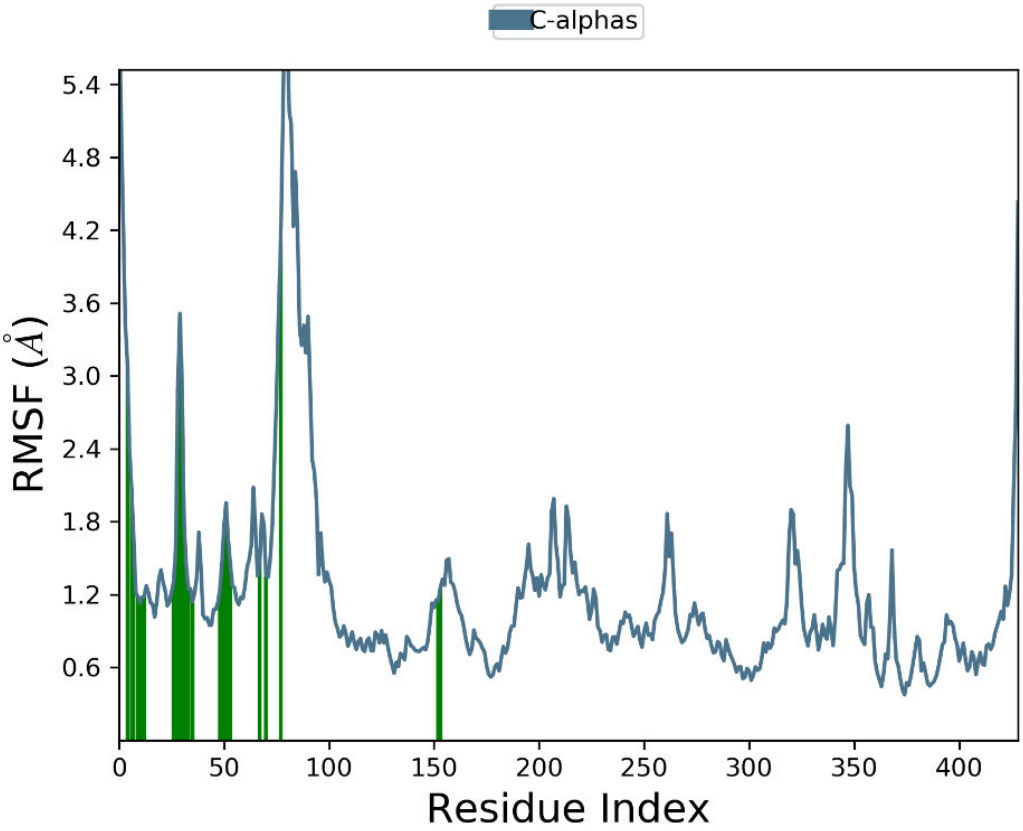

Figure 8.12: The fluctuations of each amino acid residue in the protein structure are calculated and represented as a function of time.

There are four main types of interactions, also referred to as "contacts": hydrogen bonds, hydrophobic, ionic, and water bridges. There are additional subtypes for each type, which are explained in the 'Simulation Interactions Diagram' panel. Hydrogen bonds play a vital role in the binding of ligands, impacting the specificity of drugs, their metabolism, and their adsorption. There are four subtypes within this category: backbone acceptor, backbone donor, side-chain

acceptor, and side-chain donor. Accurate measurements of distance and angle are used to assess the H-bond between the protein and ligand, offering valuable insights into the dynamics of their interaction. There are three subtypes in this category: π-Cation, π-π, and other non-specific interactions. The geometric criteria for each subtype are determined by specific proximity and orientation requirements, typically involving the presence of a hydrophobic amino acid and an aromatic or aliphatic group on the ligand. Ionic interactions, or polar interactions, occur when atoms with opposite charges are close to each other. There are two distinct types, which can be distinguished based on whether the contact is facilitated by the protein backbone or the side chains. In addition, the study of protein-metal-ligand interactions involves closely observing the coordination of a metal ion with the heavy atoms of both the protein and the ligand within a specific distance. Water bridges are formed when water molecules create hydrogen-bonded connections between the protein and ligand. The geometric criteria for these interactions have more relaxed parameters compared to traditional H-bond definitions, allowing for a nuanced and thorough evaluation. The stacked bar charts in the graphic are normalised based on the trajectory, representing the proportion of simulation time that each given interaction type is sustained. Values greater than 1.0 may occur, suggesting situations where a protein residue forms multiple interactions of the same subtype with the ligand.

The graphic shows the progression of the Root Mean Square Deviation (RMSD) of the protein and ligand throughout the simulation, as seen in Figure 8.13. The y-axis on the left side shows the Root Mean Square Deviation (RMSD) values of the protein, which provide insights into the structural variations from the reference frame backbone. Monitoring the root-mean-square deviation (RMSD) of proteins provides valuable insights into the variations in structural conformation throughout the simulation. Fluctuations observed around the thermal average structure towards the end of the simulation suggest that equilibration is taking place. Small, globular proteins are considered satisfactory if they show changes within the range of 1-3 Å. However, significant changes may indicate important conformational shifts. The y-axis on the right side of the graph represents Ligand RMSD, which provides valuable insights into the stability of the ligand in relation to the protein and its binding pocket. The 'Lig fit Prot' values indicate the root mean square deviation (RMSD) of the ligand when it is aligned with the protein backbone of the reference frame. If the RMSD values of the ligand are much higher than those of the protein, it suggests that the ligand may have diffused away from its initial binding site. Understanding the stability of the ligand within the binding pocket and its behaviour in relation to the protein during the simulation is crucial for this observation.

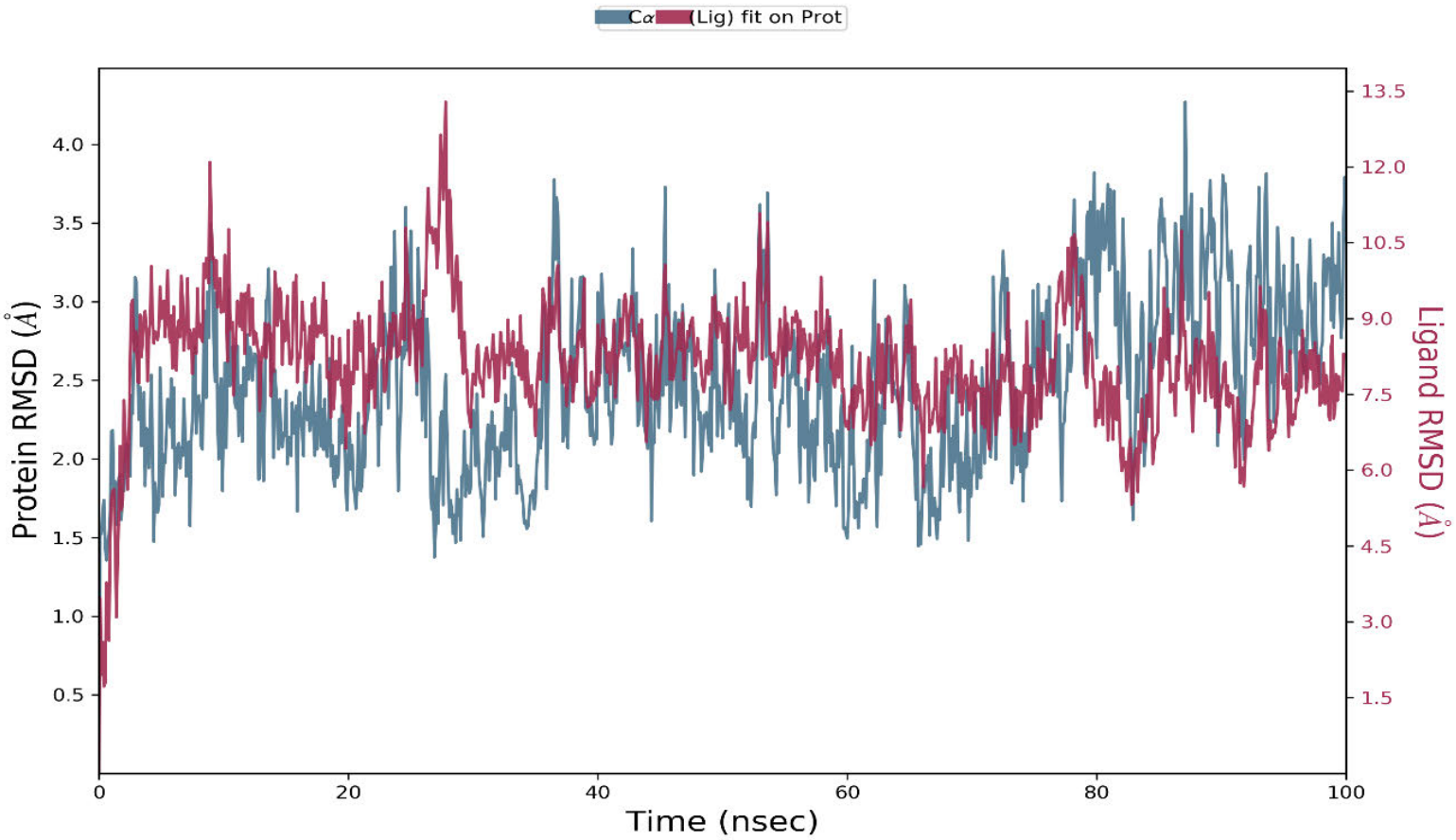

Figure 8.13: The average distance between the atoms of a particular structure and a reference structure, providing insights into the structural stability and conformational changes occurring over time.

8.5 DISCUSSION

When dealing with gonococcal infections, the main objective of β-lactam antibiotics is to target penicillin-binding protein 2 (PBP2) in *N. gonorrhoeae* with precision. Certain strains of *N. gonorrhoeae*, such as FA6140, exhibit the addition of an aspartate residue following the 345th position in the PBP2 protein. According to a source, there are 4–8 additional changes that occur alongside this insertion, referred to as As*p*-345a. Nevertheless, the impact of these mutations on the protein's structural integrity remains uncertain. The crystal structure of PBP2 from the penicillin-resistant strain FA6140 (Bellini et al., 2019) to perform the analysis on the target protein was used. This strain shows four mutations in the C-terminal region of the protein, resulting in a notable 5-fold reduction in the rate of penicillin binding compared to the standard type. Through extensive kinetic investigations, it has been discovered that the acylation rate experiences a significant decrease due to two specific mutations, namely P551S and F504L (Powell et al., 2009). These mutations also lead to a reduction in the enzyme's thermal stability, as evident from the melting curves. Furthermore, previous studies have highlighted the impressive antibacterial and antifungal properties of rutin, enabling it to effectively combat a wide range of harmful microorganisms. By exploring molecular interactions and binding affinities, the aim is to uncover promising inhibitors that can effectively hinder the activity of penicillin-binding protein-2. Additional investigation has unveiled the remarkable ability of rutin to efficiently attach to the PBP2 of FA19 *Neisseria gonorrhoeae*, a vital catalyst in

the creation of the microbial cellular barrier. Prior research has shown the efficacy of rutin in fighting gram-negative bacteria, indicating a possible mechanism that involves its interaction with PBP2 (Arima et al., 2002; Kosanić et al., 2012). By studying the FA6140 and FA19 PBP2 proteins, one can gain a deep understanding of how rutin affects important proteins in both penicillin-resistant and penicillin-susceptible strains of *N. gonorrhoeae*. This study uncovers the complex interactions between these proteins and rutin. Having a deep understanding of the intricate molecular mechanisms involved in antibiotic resistance is of utmost importance. These specific mutations have been discovered to interfere with the active site, resulting in challenges for antibiotics to bind or for essential conformational changes to take place during β-lactam antibiotic acylation. Furthermore, certain phenolic compounds have demonstrated exceptional antibacterial activity, in addition to their widely recognised antioxidant properties (Bellini et al., 2019). A selection of five compounds was made to investigate the interactions between the penicillin-resistant FA6140 PBP2 and certain phytocompounds with antibacterial properties. The selection of these compounds was based on their prior *in vitro* antibacterial activity. The study aimed to gain insights into the antibacterial mechanisms of these compounds, which have been observed to affect the permeability of cell membranes, interact with enzymes through hydrogen bonding to influence intracellular functions, and modify the rigidity of cell walls. These interactions with the cell membrane can result in damage to its integrity, as shown in previous studies (Fakoya et al., 2020;

Kosanić et al., 2012; Tungmunnithum et al., 2018; Yakobi et al., 2023). Advanced analytical techniques were employed to simulate the binding process and evaluate interactions. Extensive molecular docking analyses were conducted on all five target compounds against the FA6140 PBP2 enzyme's 3eqv [A]. According to the experimental findings, it was noticed that rutin exhibited the lowest energy consumption during the binding process. After that, quercetin, ferulic acid, *p*-coumaric acid, and protocatechuic acid were observed in that specific sequence. These findings provide further support for previous *in vitro* research, suggesting that specific flavonoids, like quercetin and rutin, demonstrate superior antibacterial efficacy in comparison to other compounds. Rutin, with its molecular formula $C_{27}H_{30}O_{16}$, exhibits remarkable antioxidant properties, effectively diminishing various oxidising species like superoxide, peroxyl, and hydroxyl radicals (Abdelrheem et al., 2020). In addition, it shows noteworthy interactions with the FA6140 PBP2 enzyme. This comprehensive analysis deepens the comprehension of the antibacterial properties of these compounds, particularly in regards to the penicillin-resistant FA6140 PBP2 enzyme. Moreover, these compounds exhibit pharmacological properties, including anti-cancer, antibacterial, and anti-inflammatory effects. The research indicates that both quercetin and rutin have the ability to bind to the substrate binding sites of both FA19 and FA6140 PBP2 enzymes. The FA6140 PBP2 binding site shows a significant affinity for rutin, as indicated by its remarkable binding energy of -8.1 kcal/mol. Despite its strong binding affinity, rutin does not possess drug-like

properties, according to Lipinski's rule. However, quercetin, with a Log Po/w of 1.63 and no druglikeness violations, seems to be a more encouraging prospect. In previous studies, it was noted that caulerpin adheres to Lipinski's rule and has a satisfactory ADMET profile. There is potential for this natural compound to be utilised in drug development in the future (Abdelrheem et al., 2020). In line with this investigation, this research showcases the successful blocking of the FA6140 PBP2 protein's main function by these plant compounds, leading to the prevention of the formation of penicillin-resistant *N. gonorrhoeae* bacteria. Quercetin exhibits a significant affinity of -7.6 kcal/mol for FA6140 PBP2, suggesting a strong attraction. The primary factors that govern this interaction are the energies associated with Van der Waals and electrostatic forces. Acquiring three-dimensional structures through crystallography can be quite a complex process due to various factors such as crystal packing and static disorder. Building upon the previous laboratory research, it was discovered that flavonoids like quercetin and rutin have shown improved effectiveness. In the latest study, it was found that protocatechuic acid exhibited a binding energy of -6.4 kcal/mol, indicating a strong affinity for the FA19 PBP2 binding site. It seems that protocatechuic acid has the ability to disrupt the main function of the FA19 PBP2 protein, which could hinder the growth of penicillin-resistant gonorrhoeae in cells. Studies have shown that rutin, with its remarkable antioxidant properties, has the ability to significantly mitigate the effects of different oxidising agents. In addition, it demonstrates a range of pharmacological activities, such as anti-neoplastic,

antibacterial, and anti-inflammatory properties. Upon analysing the interaction with FA19 PBP2, it was observed that both rutin and quercetin exhibited a significant affinity towards the substrate binding site. However, rutin displayed a higher binding energy of -8.1 kcal/mol. Stable complexes were observed in molecular dynamics simulations with FA6140 PBP2. After conducting a thorough analysis, it was noticed that the presence of rutin resulted in a reduction in the solvent-accessible surface area. There appears to be a favourable alignment between the ligand and the binding pocket. Flavonoids possess remarkable binding capabilities that surpass those of other plant compounds, making them highly intriguing substances for optimising leads and driving pharmaceutical development forward. Flavonoids have a strong binding affinity to the central functional units of FA6140 PBP2 and FA19 PBP2. Blind docking is a computational method that enables ligands to autonomously navigate the whole surface of a target protein without any prior knowledge of the binding location. This approach enhances the comprehension of the binding location and pharmacophoric interactions through many means. Blind docking is a method that may anticipate probable binding sites on a protein, uncovering specific areas where ligands exhibit a strong attraction. This is particularly advantageous in cases where the specific binding site is unidentified or when there are several putative binding pockets. Ligands have the ability to interact with many parts of the protein surface, rather than being limited to specific binding sites. This enables a thorough examination of the whole protein surface. This aids in

comprehending the presence of alternate binding areas outside established locations. Blind docking can reveal allosteric binding sites, which are locations that are far from the active site and can influence the activity of proteins. These websites are essential for comprehending regulatory systems. Ligands have the ability to assume diverse conformations during blind docking, which allows for a better understanding of their flexibility and the manner in which they interact with different areas of the protein. Understanding pharmacophoric interactions is essential. Blind docking enables the anticipation of unexplored connections between ligands and proteins, which might possibly unveil new binding locations or alternate binding mechanisms. Blind docking facilitated the identification of pharmacophoric characteristics, including hydrogen bonding and hydrophobic interactions, on the protein surface by analysing the docked poses of ligands. This knowledge facilitates comprehension of the essential characteristics necessary for binding. Blind docking can function as a means of confirming the accuracy of experimental results. Aligning the anticipated binding locations with existing experimental data enhances the reliability of the computational predictions. Blind docking experiments can uncover the impact of protein flexibility on ligand binding. Proteins have dynamic characteristics, and blind docking simulations can effectively reflect the protein's capacity to adapt to various ligand conformations. Blind docking is a supplementary technique to existing docking approaches that prioritise certain binding sites. Blind docking allows for a comprehensive examination, whereas focused docking studies provide specific

and detailed information about known binding areas. The simultaneous use of both methodologies enhances the comprehensive comprehension of ligand-protein interactions. The analysis of the interaction between rutin- and penicillin-suppressed PBP2, as well as the simulation of rutin- and penicillin-resistant PBP2, produced promising results. These results indicate a successful and accurate simulation. The RMSD plot showed fluctuations within the range of 1-3 Å, suggesting that there were acceptable variations for small, globular proteins. The simulation successfully reached convergence, displaying consistent RMSD values towards the end, indicating equilibration. The RMSD fluctuations remained within 1.5 Å throughout the simulation, indicating a well-equilibrated system. Peaks of Ligand RMSD were observed, which corresponded to areas of significant protein fluctuation, like the N- and C-terminal tails. The ligand RMSD displayed periodic peaks that coincided with protein fluctuations, yet it consistently stayed below 2.0 Å. This suggests that the ligand remains stable within the binding pocket. The plot clearly highlighted the protein residues that interacted with the ligand and it was observed that structural changes were gained through the distribution of secondary structure elements (SSE). Prominent ligand contacts were observed, with significant interactions in important secondary structure elements, demonstrating a strong binding pattern. The Ligand RMSF exhibited localised fluctuations, highlighting specific atom-level interactions that contribute to stable binding events. The maintenance of hydrogen bonds and hydrophobic interactions was consistent throughout, as indicated by the

normalised frequencies of binding. The timeline representation provided a concise overview of the specific contacts, while the schematic diagrams emphasised the interactions that occurred for a significant portion of the simulation time. The timeline representation revealed sustained contacts, while the schematic diagrams highlighted important interactions, giving a clear picture of the stable binding interface. The ligand torsions displayed smooth transitions, and the radial plots indicated minimal conformational strain, thus confirming the stability of the ligand in the protein-bound state. The simulation showcased promising attributes, including smooth RMSD, stable ligand behaviour, consistent interactions, and minimal conformational strain. These findings collectively suggest a dependable portrayal of the dynamics of the protein-ligand complex. In summary, these analyses offer a thorough grasp of the dynamic behaviour of the protein-ligand complex, revealing insights into structural changes, stability, and interactions during the simulation.

8.6CONCLUSION

The antibacterial effects of rutin and other phytocompounds on two strains of *Neisseria gonorrhoeae* were investigated. The binding affinity of rutin is highlighted through molecular docking analyses, suggesting its potential as an inhibitor of PBP2. Additional research on quercetin and protocatechuic acid highlights their potential as effective antibacterial agents, particularly quercetin, which exhibits properties similar to those of pharmaceutical drugs. The molecular

dynamics simulations offer a thorough comprehension of the interactions between flavonoids and PBP2. The affinity of rutin for the substrate binding site indicates its potential to inhibit penicillin-resistant *N. gonorrhoeae*, thanks to its impressive antioxidant properties. Quercetin shows great potential as a promising candidate with strong attraction and drug-like properties. Flavonoids have strong binding affinities to PBP2, indicating their potential as inhibitors to disrupt cell wall synthesis in *N. gonorrhoeae*. The research offers a thorough and insightful analysis of the protein-ligand interactions, revealing the antibacterial properties of flavonoids against penicillin-resistant *N. gonorrhoeae*. The simulation results indicating stability and accuracy, provide valuable insights for the development of effective inhibitors and the advancement of pharmaceutical interventions against infectious diseases.

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CHAPTER 9: MOLECULAR DOCKING AND STRUCTURE-ACTIVITY RELATIONSHIP ANALYSIS OF TARGET COMPOUNDS AGAINST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN AZITHROMYCIN-RESISTANT *NEISSERIA GONORRHOEAE*

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Preface

This article focuses on the crystallographic conformation of GAPDH, a key enzyme in glycolysis, derived from *Neisseria gonorrhoeae* strain NCCP11945, complexed with NAD (PDB ID: 5VMT). The aim was to elucidate the interactions and efficacy of various compounds against GAPDH to combat azithromycin-resistant strains of *Neisseria gonorrhoeae*. This article was published in *ChemistrySelect* Journal (Wiley) on February 28, 2024, in Volume 9, Issue 9. DOI link: doi.org/10.1002/slct.202303341.

9.1 ABSTRACT

The emergence of drug-resistant strains of *Neisseria gonorrhoeae* poses a significant global health challenge, necessitating the development of novel antimicrobial agents. This study focuses on the potential of phenolic compounds to target the enzyme GAPDH in *N. gonorrhoeae*, a key protein involved in glycolysis and implicated in various pathological mechanisms. Among the compounds evaluated, quercetin demonstrated significant binding affinity to *N. gonorrhoeae*-derived GAPDH. Structural integrity assessments using Procheck software and molecular docking simulations confirmed the binding capacity of quercetin. Molecular dynamics simulations further explored the stability and flexibility of the quercetin-*N. gonorrhoeae* GAPDH complex. The results revealed interactions between quercetin and specific amino acid residues, suggesting potential binding sites crucial for antimicrobial action. This information provides valuable insights into the development of quercetin-based therapeutics targeting drug-resistant *N. gonorrhoeae*, addressing the urgent need for novel antimicrobial agents.

9.2 INTRODUCTION

Azithromycin, a potent oral antibiotic, was initially approved for its efficacy against chlamydia infections and subsequently for treating *Neisseria gonorrhoeae* due to its commendable activity against this pathogen. However, in recent years, the susceptibility of *N. gonorrhoeae* to azithromycin has decreased significantly, with the emergence of azithromycin-resistant strains (Jefferson et al., 2021; Yakobi & Pooe, 2022). This rapid development of antimicrobial resistance has greatly hindered the ability to manage gonorrhoeae infections effectively. Multidrug-resistant bacteria necessitate the development of innovative antimicrobial agents to combat these resilient pathogens (Jacobsson et al., 2018; Unemo et al., 2021). Globally, third-generation cephalosporins (ceftriaxone and cefixime) are the primary pharmacological agents used for managing gonococcal infections, either as monotherapy or in combination with macrolides like azithromycin. Reports indicate increasing resistance to azithromycin, cephalosporin, and fluoroquinolones in *N. gonorrhoeae* (Hummell & Kirienko, 2020; Lim et al., 2021; Lin et al., 2021). The emergence of ceftriaxone-resistant strains poses a significant challenge to the efficacy of this antimicrobial agent, which is currently the sole first-line treatment option for gonorrhoeae. The limited armamentarium of antimicrobial agents for managing drug-resistant *N. gonorrhoeae* highlights the urgent need for novel antimicrobial agents. Given the rising prevalence of *N. gonorrhoeae* infections and the recurrent syndromic management of STIs, it is crucial to discover novel agents

with dual efficacy against *N. gonorrhoeae* (Thangamani et al., 2016; Unemo, 2015; Yakobi & Pooe, 2022). Identifying and characterizing novel protein targets is essential to pave the way for developing new antimicrobial agents (Magiorakos et al., 2017). GAPDH, an enzyme central to glycolysis, plays a pivotal role in numerous metabolic pathways in both prokaryotic and eukaryotic organisms. GAPDH catalyses the oxidation of glyceraldehyde-3-phosphate (GAP) to 1,3-bisphosphoglycerate while reducing NAD+ to NADH (Butera et al., 2019; Hillion et al., 2017). Evidence suggests that *Neisseria* GAPDH can manipulate host cell mechanisms to favour the pathogen. Under low oxidant conditions, certain active site SH-groups in GAPDH undergo oxidation, forming sulfenic acid derivatives (S-OH), which can catalyse the hydrolysis of acylphosphates, a reaction known as acylphosphatase (Lazarev et al., 2020). The enzyme, possessing SH- and S-OH groups, exhibits dual functionality as a dehydrogenase and an acylphosphatase. Slight oxidation of GAPDH leads to the dissociation of oxidative and phosphorylative events in glycolysis, reducing cellular ATP levels (Gupta & Carroll, 2014; Ivanov et al., 2021). GAPDH has attracted attention due to its involvement in apoptosis and the pathogenesis of various neurodegenerative disorders. Oxidation of GAPDH may play a key role in diverse pathological mechanisms, and protecting the enzyme from oxidation might prevent certain pathologies. The cumulative findings from various biological investigations support the viability of GAPDH as a therapeutic target against drug-resistant *N. gonorrhoeae* using phenolic compounds (Butera et al., 2019; Chen et al., 2020;

Gao et al., 2020; Tian et al., 2016). To identify novel lead compounds that selectively target GAPDH, a strategy for selecting compound libraries based on the structural analysis of *N. gonorrhoeae* GAPDH was developed. Amidst the increasing antimicrobial resistance, *N. gonorrhoeae* presents a significant challenge, particularly due to the rise of azithromycin-resistant strains. This study aims to utilize sophisticated molecular docking techniques and perform an extensive analysis of structure-activity relationships for potential compounds targeting GAPDH, a crucial enzyme in the metabolic pathway of *N. gonorrhoeae*. Recent investigations have clarified that *N. gonorrhoeae* has developed resistance mechanisms against azithromycin, necessitating novel therapeutic interventions. To screen for new drug candidates, the binding affinity of five target phytochemicals—ferulic acid, *p*-coumaric acid, protocatechuic acid, quercetin, and rutin—with *N. gonorrhoeae*-derived GAPDH was evaluated. Molecular docking simulations were used to assess the potential effectiveness of these interactions, aiming to discover novel agents to combat drug-resistant *N. gonorrhoeae*.

9.3 MATERIAL AND METHODS

Please refer to chapter 3, subsection 3.8 – 3.11, page 109 – 116 for a detailed material and methods protocol.

9.4 RESULTS

9.4.1 Identification of allowed and disallowed regions of protein backbone conformation

The structural integrity of the *N. gonorrhoeae* GAPDH model was meticulously evaluated using Procheck software. The analysis of the Ramachandran plot revealed a predominant central region encompassing 91.9% of the residues, accompanied by a minor permissible region of 8.1%. Notably, no residues fell within regions considered restrictively or permissively unfavourable in terms of conformational properties. Among the extensive Ramachandran analyses, only a scant 21 designated residues out of a total of 2592 were identified. Chi1-chi2 plots further indicated the presence of 24 specific residues, constituting a small proportion of the overall 1236 residues, with five instances demonstrating exceptional traits regarding side-chain parameters. None of these parameters were situated in the interior, and no evidence of degradation was observed. After thorough analysis of residue characteristics, the highest discrepancy was found to be 5.5. Additionally, no unfavourable intermolecular interactions were noted, while deviations in bond length and angle were determined to be 8.7. The dihedral angle of the G-factors was observed at -0.20, with a covalent radius of 0.50 and

a comprehensive G-factor value of 0.09. Planar groups adhered strictly to predetermined standards, with no instances of nonconformance. The graphical representation highlighted the correlation between tridimensional atomic configuration and the respective linear sequence of amino acids. Analysis of the obtained outcomes against pre-established structural benchmarks revealed that 61.93% of amino acid residues exhibited a mean 3D-1D score equal to or greater than 0.1. Moreover, a substantial fraction, amounting to no less than 80% of amino acids, exhibited a 3D/1D profile score of 0.1 or higher, as depicted in Figure 9.1. The overall quality assessment resulted in a global quality score of 94.8345 and an average probability value of 5.35938, indicating a high level of quality in the analysed sample.

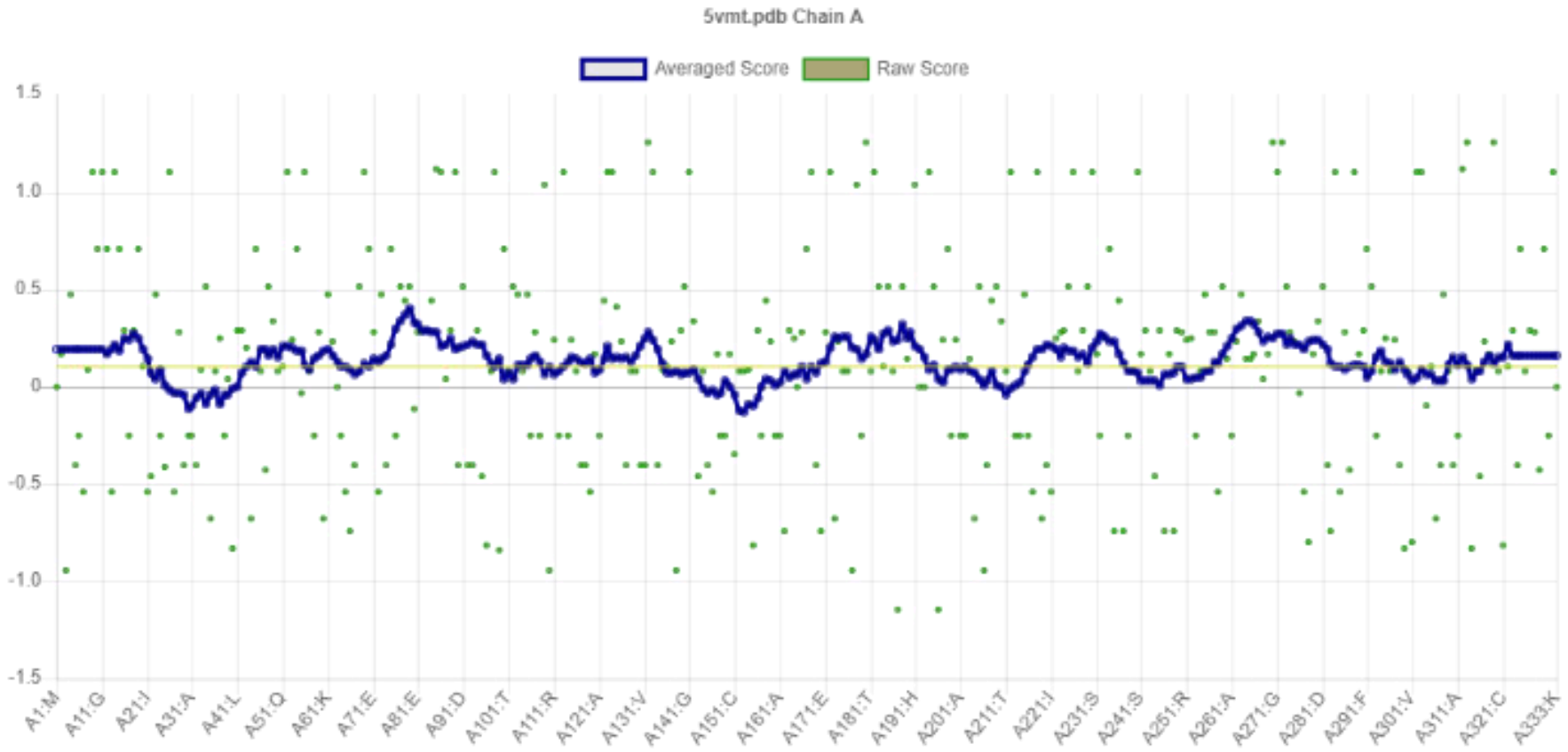

Figure 9.1: Comparing the spatial arrangement of atoms in the protein's structure with the linear sequence of amino acids encoded by its genetic sequence.

9.4.2 Molecular docking analysis using pyrx and schrödinger

In this study, PyRx software was used to conduct molecular docking analyses to evaluate the binding affinity of specific phytochemicals with the *N. gonorrhoeae* GAPDH model. The main goal was to evaluate the binding strength of the mentioned chemical compounds. The investigation employed computational techniques to model the binding mechanism and analyse the resulting intermolecular association. For this study, molecular docking analyses on a wide range of five target compounds using the 5vmt [A] protein was performed, see crystal structure in Figure 9.2. Based on the experimental findings, it appears that the interaction between the 5vmt [A] protein and rutin led to the least amount of energy used during binge-eating. Following the previously mentioned information, there was a consecutive appearance of quercetin, ferulic acid, protocatechuic acid, and *p*-coumaric acid. The interactions between ligands and hydrophobic residues play a significant role in determining the binding energy of proteins. The well-known phenomenon of amino acid residues' hydrophobic nature is characterised by their inclination to exhibit repulsive behaviour towards polar functional groups and water molecules. The illustration shows the molecular interaction between rutin and *N. gonorrhoeae* GAPDH, leading to the attraction of the ligand's non-polar groups. This phenomenon describes the gathering of substances that repel water in a watery setting. Based on the findings, it appears that the flavonoid rutin forms connections with *N. gonorrhoeae* GAPDH through hydrogen bonding and hydrophobic interactions. In addition,

the research shows that substances that are not soluble in water tend to come together in a water-based environment due to the hydrophobic effect.

In this study, molecular docking analyses were conducted using both PyRx and Schrödinger software to investigate the binding affinities and interactions between different target compounds and the 3eqv [A] protein, which represents GAPDH from *Neisseria gonorrhoeae*. The compounds evaluated included protocatechuic acid, *p*-coumaric acid, ferulic acid, quercetin, and rutin. In the PyRx docking analysis, various target compounds were assessed for their binding affinity to the GAPDH protein. Protocatechuic acid exhibited a docking score of -6.4 kcal/mol, forming a hydrogen bond interaction with ASN-33, along with additional interactions with ASN-8, LEU-35, and THR-97. Similarly, *p*-coumaric acid showed a docking score of -6.3 kcal/mol, engaging in a hydrogen bond interaction with ASN-8 and other interactions with PHE-99 and LEU-35. Ferulic acid demonstrated a docking score of -6.5 kcal/mol, with a hydrogen bond interaction observed with AS*P*-34 and other interactions with THR-97 and LEU-35. Quercetin exhibited a docking score of -7.6 kcal/mol, forming hydrogen bond interactions with ASN-315, CYS-151, THR-211, and ARG-234, along with other interactions with CYS-151, HIS-178, THR-181, ALA-232, and THR-211. Rutin displayed the highest docking score of -9.5 kcal/mol, establishing hydrogen bond interactions with CYS-96, ILE-13, ASN-315, THR-152, ARG-234, and SER-210, as well as other interactions with GLU-316, ARG-12, and GLY-182.

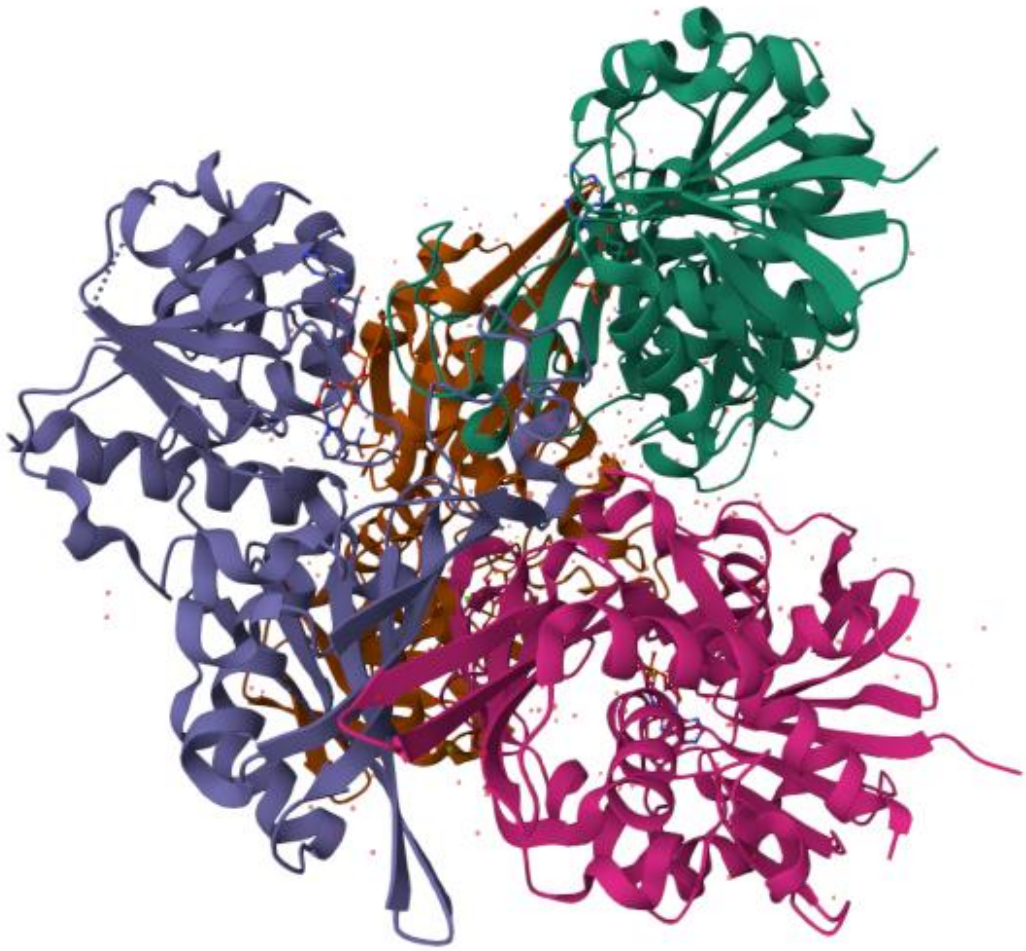

Figure 9.2: Crystal structure of a glyceraldehyde-3-phosphate dehydrogenase from *Neisseria gonorrhoeae* bound to NAD.

The green, purple, pink, and orange domains represent the different subunits within each subunit of the tetrameric GAPDH enzyme, with each domain contributing to the structural stability or interact to form the active site. Furthermore, in GAPDH structures, the active site is generally located near the NAD binding site, which might be visualized here by close proximity of subunits and specific residues within one domain.

In contrast, the Schrödinger docking results revealed similar binding affinities and interaction patterns. Protocatechuic acid exhibited a docking score of -6.2 kcal/mol, forming hydrogen bond interactions with ASN-33 and ASN-8, along with other interactions with LEU-35 and THR-97. *p*-Coumaric acid showed a docking score of -6.1 kcal/mol, engaging in a hydrogen bond interaction with ASN-8 and additional interactions with PHE-99 and LEU-35. Ferulic acid demonstrated a docking score of -6.3 kcal/mol, with a hydrogen bond interaction observed with AS*P*-34 and other interactions with THR-97 and LEU-35. Quercetin exhibited a docking score of -7.4 kcal/mol, forming hydrogen bond interactions with ASN-315, CYS-151, THR-211, and ARG-234, along with other interactions with CYS-151, HIS-178, THR-181, ALA-232, and THR-211. Rutin displayed a docking score of -9.2 kcal/mol, establishing hydrogen bond interactions with CYS-96, ILE-13, ASN-315, THR-152, ARG-234, and SER-210, as well as other interactions with GLU-316, ARG-12, and GLY-182. These results provide valuable insights into the potential binding affinities and mechanisms of action of these compounds with the GAPDH protein.

The docking analysis results provide confirmation of the molecular basis for the interaction and arrangement of the specific phytochemicals with the binding sites of *N. gonorrhoeae* GAPDH. This analysis aimed to predict how well the compounds would bind to the protein's active site and their orientation within it. Based on the findings, it is clear that the phytochemicals have the ability to form

multiple hydrogen bonds and non-covalent interactions with the important functional residues of the protein being studied. Extensive research has shown that rutin exhibits a higher binding affinity towards the target protein compared to other compounds. The molecular entity demonstrates a high level of expertise in establishing typical hydrogen bonding interactions with important functional amino acid residues, including CYS-96, ILE-13, ASN-315, THR-152, ARG-234, and SER-210. This results in a docking score of -9.5 kcal/mol. Based on the experimental results, it is evident that rutin shows a strong attraction to the GLU-316 amino acid residue, resulting in the formation of a Pi-Anion bond. In addition, a unique bond was detected at ARG-12, while a specific hydrogen bond was observed at GLY-182, see Figure 9.3.

In addition, two Pi-Alkyl interactions were found at ARG-12, as shown in Figure 9.4. The lengths of bonds within a molecule provide important insights into the strength and longevity of these bonds. When examining rutin, analysing the bond lengths can offer valuable information about the presence of stable covalent bonds in the molecule. Significant deviations from standard bond lengths may suggest alterations in structure or interactions with other molecules. For instance, when the atoms in rutin undergo changes in their distances as they bind to a target, it could suggest alterations in its shape or specific interactions. Hydrogen bonding plays a crucial role as a noncovalent interaction in biological systems. This

phenomenon involves the interaction between a hydrogen atom and an electronegative atom, such as oxygen or nitrogen, from a separate molecule.

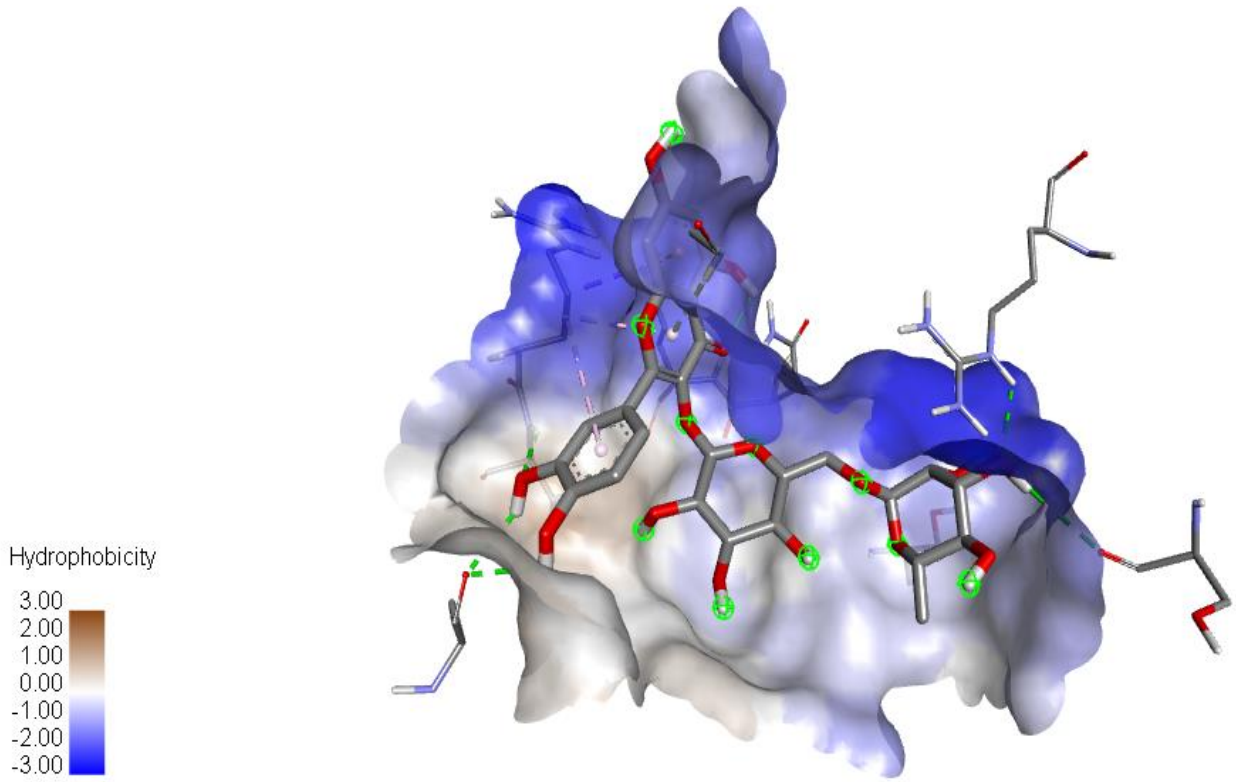

Figure 9.3: The molecular interaction between rutin and *N. gonorrhoeae* GAPDH involves a complex interplay of various forces and interactions that govern their binding affinity and stability. Rutin, a phytochemical compound, interacts with *N. gonorrhoeae* GAPDH primarily through non-covalent interactions, such as hydrogen bonding and hydrophobic interactions on CYS-96, ILE-13, ASN-315, THR-152, ARG-234, and SER-210, GLU-316, ARG-12, and GLY-182 amino acids.

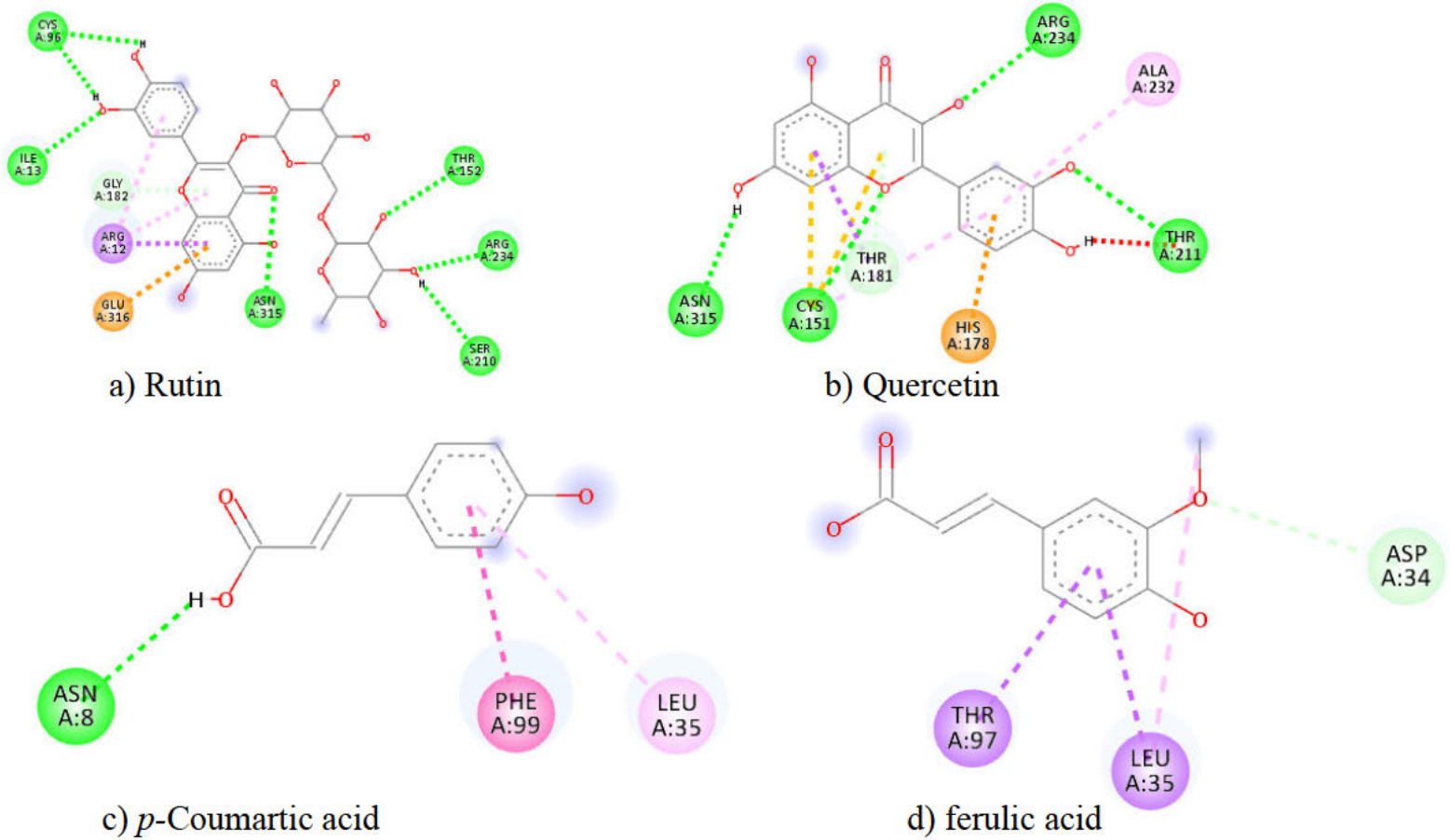

Figure 9.4: A visual depiction of the connections between a specific protein and chosen substances in a two-dimensional format.

The detection of hydrogen bonds between rutin and its target demonstrates a high level of accuracy in identifying and binding. The strength and arrangement of these hydrogen bonds play a crucial role in determining the stability of the complex and can provide insights into the compound's biological activity. Hydrophobic interactions happen when nonpolar groups gather in a hydrophobic setting, typically avoiding any interaction with water molecules. They play a significant role in the processes of molecular recognition and binding. If rutin interacts hydrophobically with the target, it suggests that the chemical can bind to specific sites. Understanding the potency of rutin's attraction and selectivity towards its biological target is essential.

Figure 9.5 shows the interpolated charge solvent surfaces of the binding site between rutin and *N. gonorrhoeae* GAPDH, emphasising the notable differences in charge. The binding affinity of Quercetin towards the specified protein was found to be significant. It formed strong interactions with the crucial functional amino acids, involving conventional hydrogen bonds at specific locations. In addition, there were two Pi-Sulfur bonds found at the CYS-151 amino acid, a Pi-cation bond at HIS-178, and a Pi-Sigma and Pi-donor hydrogen bond at the THR-181 amino acid. At ALA-232 and CYS-151, some interesting pi-alkyl interactions were found. However, it was also noticed that an unfavourable donor-donor interaction at THR-211.

The thermodynamic stability of the interaction was evaluated by calculating the docking score, which yielded a value of -7.6 kcal/mol.

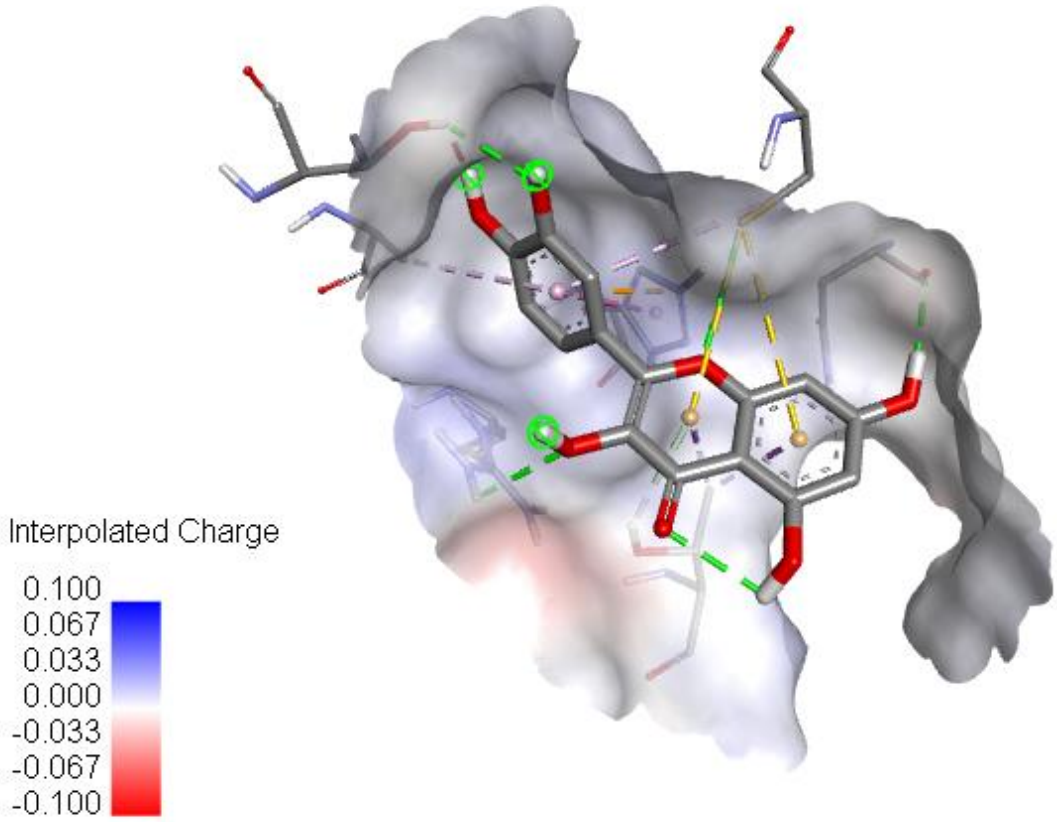

Figure 9.5: The charge surface of the binding pocket residues in the interaction between rutin and *N. gonorrhoeae* GAPDH, interactions on CYS-96, ILE-13, ASN-315, THR-152, ARG-234, and SER-210, GLU-316, ARG-12, and GLY-182 amino acids.

Based on the findings, it is evident that *p*-coumaric acid exhibited a hydrogen bonding interaction with ASN-8, a Pi-Pi Stacking interaction with PHE-99, and a Pi-Alkyl interaction with LEU-35.

The thermodynamic stability of the interaction was evaluated by calculating the docking score, which yielded a value of -6.3 kcal/mol. There is a fascinating molecular association between ferulic acid and *N. gonorrhoeae* GAPDH. This association involves a carbon-hydrogen linkage at the amino acid residue AS*P*-34, as well as two Pi-Sigma bonds at THR-97 and LEU-35, and one Alkyl bond at LEU-35. The complex of ferulic acid and *N. gonorrhoeae* GAPDH demonstrates a binding affinity of 6.5 kcal/mol, which suggests a high level of thermodynamic stability. Observations were made regarding the binding of protocatechuic acid to the target protein. A hydrogen bonding interaction was found at the ASN-33 residue, while an unfavourable donor-donor bond was observed at the ASN-8 residue. Additionally, two Pi-Sigma bonds were identified at the LEU-35 and THR-97 residues. The complex formed between protocatechuic acid and the *N. gonorrhoeae* GAPDH exhibited a binding affinity of 6.4 kcal/mol, indicating its thermodynamic stability.

Understanding the Ki value holds paramount importance in evaluating the efficacy of prospective compounds aimed at inhibiting a specific enzyme, such as GAPDH in azithromycin-resistant *Neisseria gonorrhoeae*. The Ki value denotes the equilibrium constant governing the dissociation process of the enzyme-inhibitor complex. It derives from the ratio of rate constants governing complex production (association) and dissociation. A diminished Ki value signifies heightened binding affinity between the inhibitor and the enzyme.

Practically, a low Ki implies that the inhibitor adeptly curtails enzyme activity, even at low concentrations. The study involved scrutinizing molecular docking outcomes and establishing structure-activity relationships to estimate Ki values of various candidate drugs. The selection of quercetin for further experimental validation and optimization in the drug development pipeline stemmed from this insightful comprehension of Ki values.

9.4.3 Molecular dynamics simulations analysis

An extensive analysis was conducted on Quercetin, which included a thorough examination of molecular dynamics (MD) simulations using the Desmond package. This investigation sought to examine potential binding sites in regions A. Interestingly, rutin was excluded from this section of analysis because it does not possess druglikeness properties. The Root Mean Square Fluctuation (RMSF) analysis of a protein-ligand complex measures the level of flexibility or mobility displayed by individual atoms within a molecular system during a simulation. Atoms that have higher L_RMSF values show greater flexibility or mobility throughout the simulation, whereas atoms with lower values display significantly higher stability. Higher readings of root mean square fluctuation (RMSF) could indicate regions in the protein or ligand that undergo significant structural changes or exhibit greater flexibility. The peaks in L_RMSF suggest the existence of regions involved in binding or unbinding processes, or areas with increased conformational dynamics. Atoms 6, 19, and 21 in the protein have elevated

L_RMSF values, suggesting more flexibility in comparison to the other atoms. The atom 6 in the ligand exhibits a significantly higher L_RMSF value, suggesting a greater degree of mobility. The percentage of interaction for each frame is determined by calculating the RMSF (root mean square fluctuation) of individual atoms in the protein relative to the protein's overall structure using the 'Lig_wrt_Protein' columns.

This measurement quantifies the degree of deviation of each atom in the protein from its average position during the simulation. Higher values suggest greater flexibility or mobility in specific areas. The numbers 'Lig_wrt_Ligand' represent the root-mean-square fluctuation (RMSF) of individual atoms in the ligand compared to the overall structure of the ligand. This provides information on the flexibility or mobility of the ligand atoms during the simulation. Once again, higher numbers indicate increased mobility.

From the analysis of 332 amino acids, it was found that 60 of them exhibited interactions with quercetin, as revealed by the data on specific amino acid residues. From the analysis, it is evident that certain amino acid residues, including GLY_9, GLY_11, ARG_12, and others, have direct interaction with the ligand. This information provides comprehensive details about the chain, amino acid type, ligand contact status, various spatial metrics, and the B-factor, which evaluates thermal mobility. The spatial measurements (Cα, backbone, sidechain, and all_heavy) offer valuable insights into the distances between

various atoms within the residues. The B-factor values offer valuable information regarding the flexibility and thermal mobility of each residue.

Increased B-factor values suggest a greater degree of mobility or flexibility. The residue GLY_9, with a B-factor value of 32.615, exhibits a moderate level of flexibility. There was a close proximity between the residue GLY_9 in chain A and the ligand, with Cα, backbone, sidechain, and all_heavy distances measuring around 0.8 Å. The B-factor value of 32.615 indicates a moderate level of flexibility. Did chain A come into contact with the ligand ARG_12? The spatial characteristics of GLY_9 are similar, but its B-factor is slightly lower at 30.225. There was a direct interaction between the residue GLU_22 in chain A and the ligand. The spatial measurements of certain elements were found to be larger when compared to others, specifically GLY_9 or ARG_12. Additionally, these elements exhibited a higher B-factor (35.618), suggesting an increased level of flexibility. There was an interaction between the ligand and a specific amino acid residue in the protein chain. This interaction had larger spatial dimensions and a higher B-factor value (34.615). GLY_182 in chain A was contacted by the ligand. The spatial measurements are significantly elevated, suggesting a considerable degree of flexibility as indicated by the B-factor of 35.012. The graph illustrating the alpha carbon RMSD, protein RMSD (Å), and ligand RMSD (Å) offers valuable insights into the fluctuations in the structure of the protein-ligand complex over time or in different conditions (Figure 9.6).

Examining the root mean square deviation (RMSD) values provided valuable insights into the dynamic behaviour of the protein-ligand complex. The decrease in the root mean square deviation (RMSD) values for both the protein and ligand suggests the formation of a stable complex. Higher RMSD values, especially in the C α and protein RMSD, suggest changes in the structure of the protein-ligand complex. Studying the fluctuations in RMSD values over time or in different environments helps in understanding the stability and dynamics of the complex. This graph illustrates the level of compatibility between the ligand and protein binding sites throughout the simulation. An observed decrease in pattern could suggest that the ligand is shifting towards a more favourable binding position, while an increasing pattern may indicate that it is exploring alternative orientations. A consistent fit indicates that the ligand maintains a steady binding conformation.

The Cα atom plays a critical role in establishing the backbone direction of every amino acid in the protein chain. In Figure 9.7, the positional changes or fluctuations of these Cα atoms in a protein structure are denoted as Cα. RMSF, measured in Angstroms (Å), is a way to assess the flexibility and mobility of atoms or groups of atoms within a molecule, specifically a protein. Understanding the RMSF values for the target Cα atoms across different residues can offer valuable insights into the fluctuation or flexibility of each amino acid residue in the protein structure. The graph displays the RMSF values for Cα atoms across

various amino acid residues. Greater flexibility or fluctuation in the positions of Cα atoms is indicated by higher RMSF values, suggesting a higher level of dynamism in those regions of the protein. The lower RMSF values indicated increased stability or rigidity in those areas. The graph provides valuable insights into the structural stability and flexibility of different regions of the protein.

Significant RMSF peaks may suggest areas of flexibility that could have functional significance as binding sites. Additionally, it was noted that fluctuations in the secondary structure over time. Peaks exceeding 45% indicated intervals when the protein exhibited a higher degree of organisation, characterised by an abundance of alpha helices or beta strands. Higher percentages of SSE indicated protein conformations that were more ordered and stable. Interestingly, there were peaks observed at 45% that indicate moments when the protein undergoes structural fluctuations or transitions to less ordered states, like coil regions. The fluctuations in SSE percentages highlight the dynamic nature of protein structure.

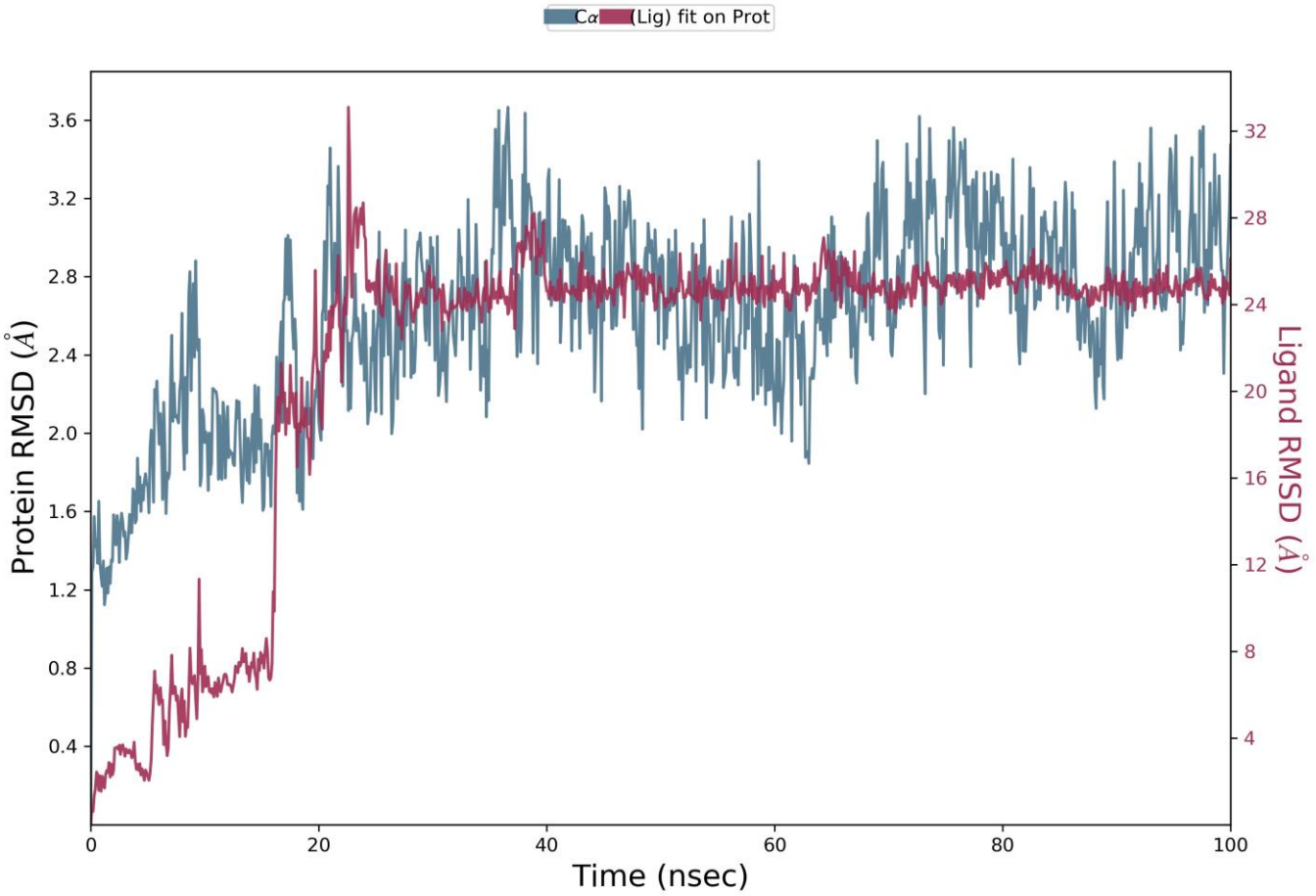

Figure 9.6: The curve illustrates the ligand's accommodation within the binding site of the protein throughout the simulation duration

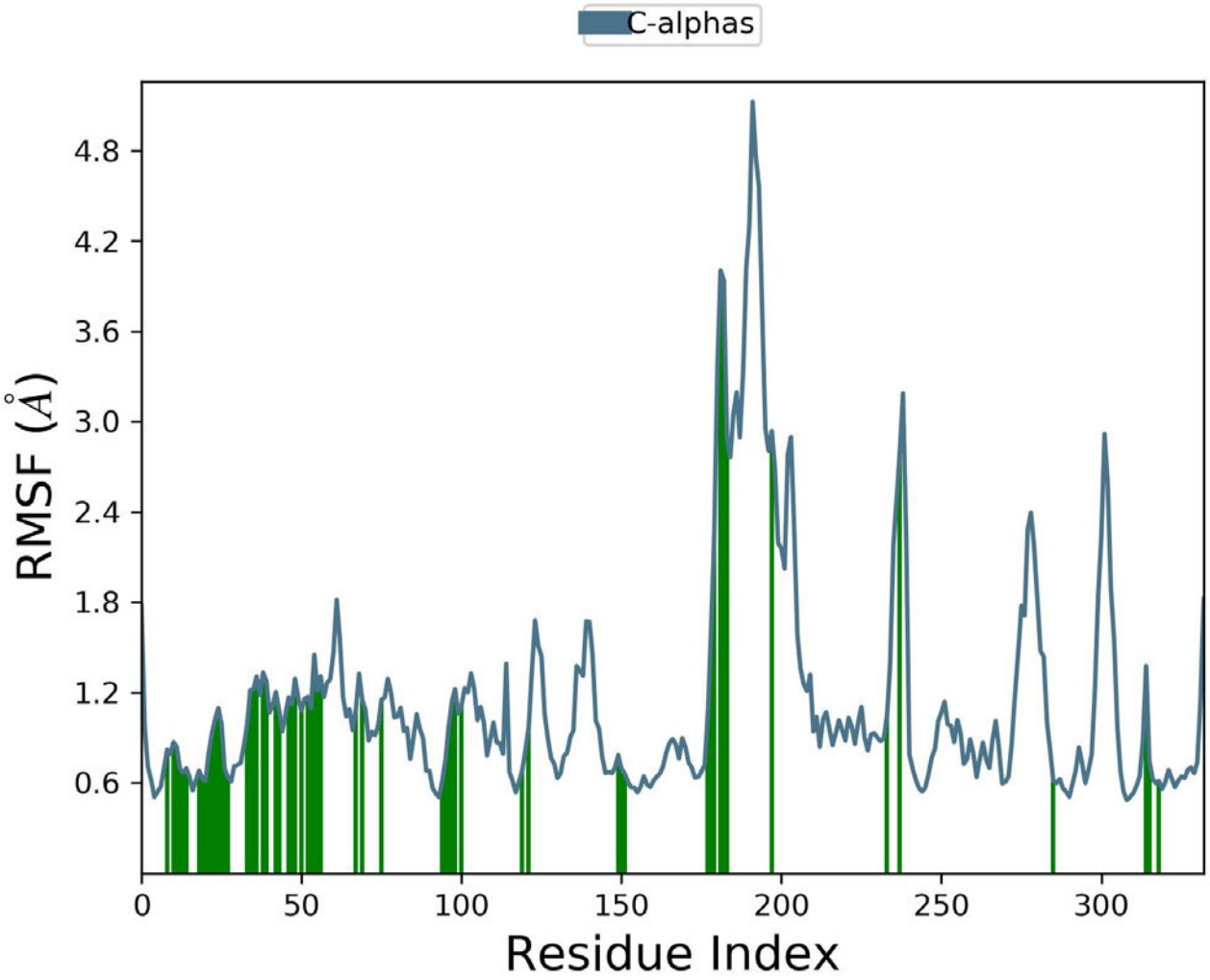

Figure 9.7: The RMSF of Cα atoms, providing insights into the flexibility of residues in the specified protein.

9.4.4 Normal Mode Analysis

Normal Mode Analysis (NMA) is a computational method used to study the dynamic behaviour of molecules, particularly proteins and nucleic acids, by examining their vibrational modes around equilibrium positions. In NMA, molecules are treated as interconnected balls and springs, where each atom represents a ball and the bonds between atoms represent springs. By solving the equations of motion for this simplified system, it's possible to predict how the molecule will vibrate in different modes, providing insights into its flexibility and stability. NMA is valuable for understanding protein dynamics, such as conformational changes and flexibility, which are essential for many biological processes, including enzyme catalysis, molecular recognition, and signal transduction.

Using internal coordinates, a thorough analysis was conducted to study the interactions between rutin and *N. gonorrhoeae* GAPDH, which is considered the optimal protein-ligand complex. Flexibility is a key factor in facilitating the interaction between biological macromolecules and the protein-ligand machinery, thus serving a vital function in these macromolecules. In order to assess the structural flexibility and molecular mobility of iMODs, the present study employed Normal Mode Analysis (NMA) by incorporating the coordinates of the docked complex. The mobility domains are displayed in the server's output as two distinct affine arrows, each with its own vibrant colour - one in red and the

other in blue. The coloured hinges throughout the chain in Figure 9.8 (b) demonstrate the intricate deformability of the structure, which is primarily influenced by the unique deformation of each residue's C atom. The value obtained from Normal Mode Analysis (NMA) was calculated by multiplying the NMA mobility by a factor of $8pi^2$. On the other hand, the B-factor value was obtained from the corresponding field in the Protein Data Bank (PDB). The B-factor, also referred to as the mean-squared displacement, represents the deviation of atomic locations from their average value. Enhanced flexibility leads to greater displacements, resulting in a decrease in electron density at the end. In Figure 9.8 (d), the server successfully computed the eigenvalue, which was determined to be 2.0312007205e-04. Furthermore, as depicted in Figure 9.8 (c), there exists a direct correlation between the eigenvalue and variance for each specific normal mode. The main-chain deformability metric can be utilised to assess a molecule's capacity to undergo deformation at the level of its individual residues. Studying areas with higher deformability provides insights into the distribution of pivot points along the chain. The research demonstrated the presence of deformability, with a peak modulus value of 1.054E+00 and a maximum deformation of 6.225E-05. As depicted in Figure 9.8 (a), the B-factor plot illustrates the stable arrangement of the docked molecules, while the B-factor graph represents the root mean square average. A colour scheme is utilised in the visual representation of the covariance matrix to indicate the presence of correlated, uncorrelated, and anti-correlated motions. Figure 9.8 (e) demonstrates how these movements are

depicted using the colours red, white, and blue, respectively. Figure 9.8 (f) displays an elastic network model illustrating the connections between the C atoms of the docked protein molecule. The model utilises springs of different stiffness levels, with the darker shades representing the springs that are more rigid.

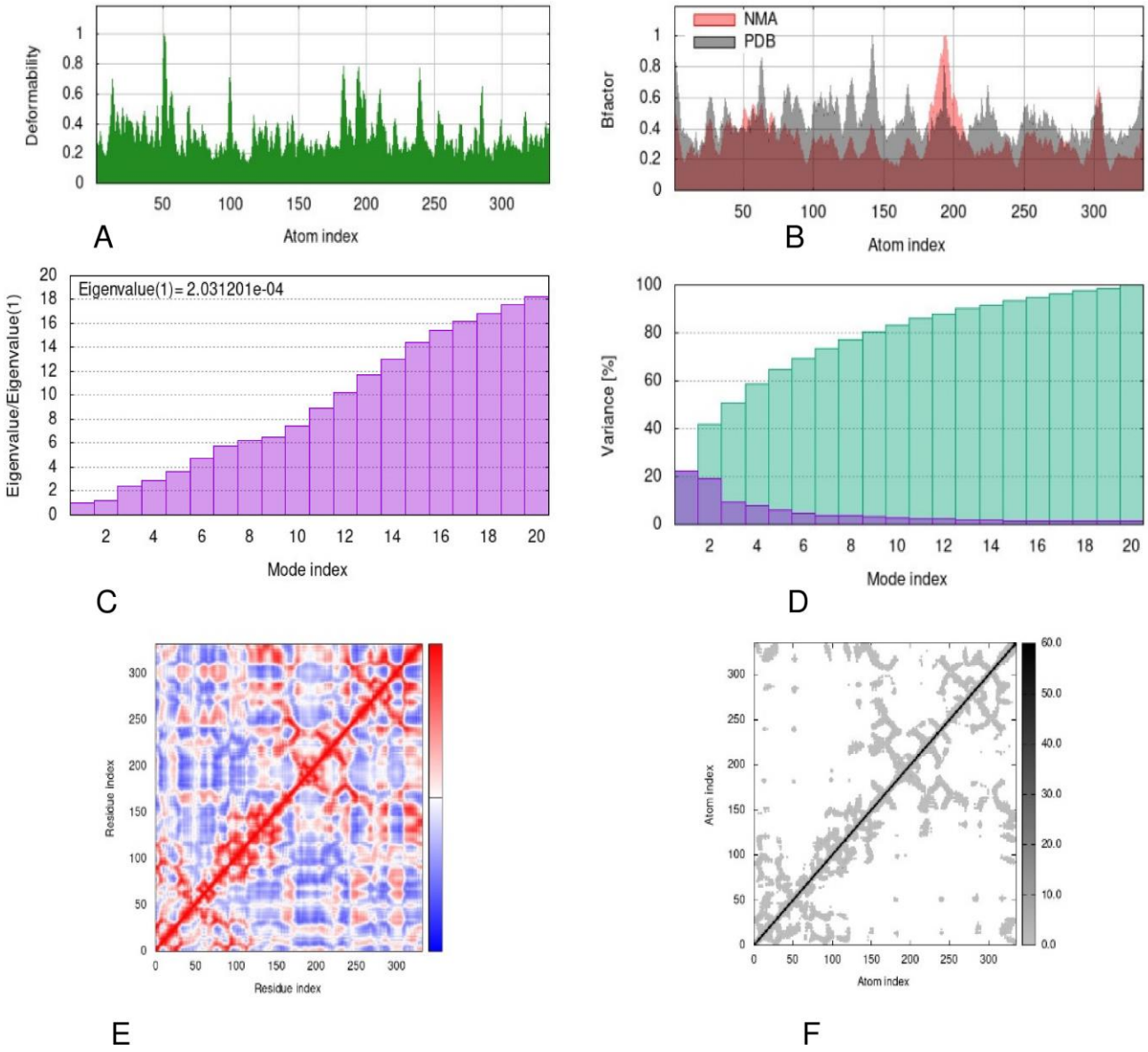

Figure 9.8: Results obtained from the iMODS Elastic Network Model (ENM) analysis, which was employed to investigate the dynamics of the protein.

The different panels represent various aspects of the analysis, providing insights into the protein's behaviour and flexibility. (a) B-Factor: Panel (a) shows the B-factor plot, which represents the atomic fluctuations or thermal motion of the protein residues. High B-factor values indicate increased flexibility or mobility of residues, while low values suggest rigidity or stability. (b) Deformability Plot: Panel (b) displays the deformability plot, illustrating regions of the protein that are prone to deformation or structural changes. Peaks in the plot indicate areas with higher deformability, which may correspond to regions involved in protein function or interaction. (c) Variance Plot: Panel (c) presents the variance plot, highlighting the contribution of each normal mode to the overall protein motion. This plot helps identify dominant modes of motion and their significance in protein dynamics. (d) Eigenvalue: Panel (d) shows the eigenvalue plot, which represents the eigenvalues obtained from the ENM analysis. Eigenvalues indicate the magnitude of motion associated with each normal mode, with higher eigenvalues corresponding to more significant motions. (e) Covariance Matrix Analysis: Panel (e) depicts the covariance matrix analysis, which reveals correlations between pairs of residues in the protein structure. This analysis helps identify residue-residue interactions and communication pathways within the protein. (f) Elastic Network Mode: Panel (f) illustrates the elastic network mode, representing the collective motion of the protein residues predicted by the ENM. This mode captures global deformations or concerted motions of the protein structure.

9.4.5 Kinetic study

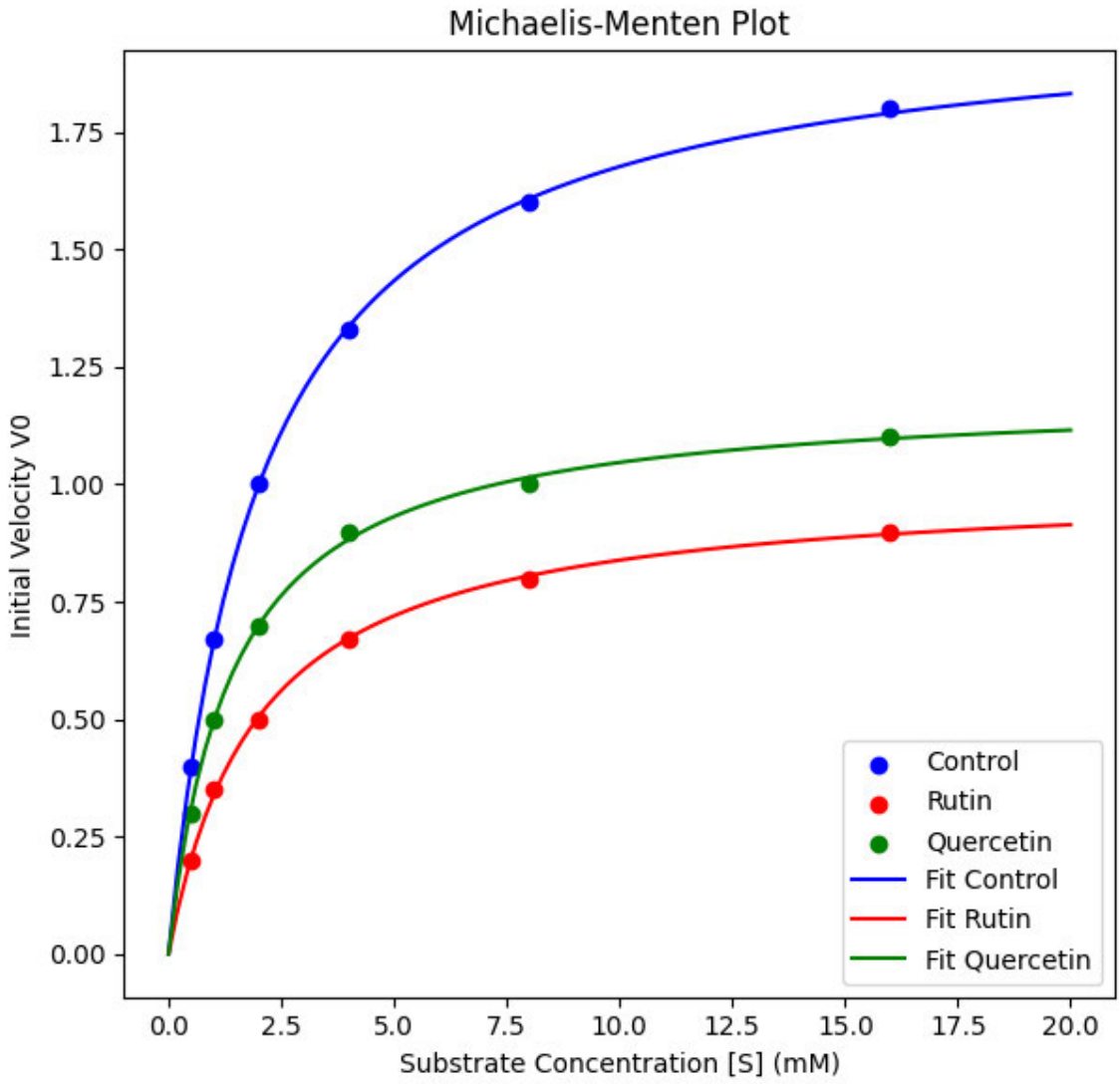

Figure 9.9: Relationship between Substrate Concentration and Initial Reaction Velocity for *N. gonorrhoeae* GAPDH: Visualizing Enzyme Kinetics and Inhibition Effects.

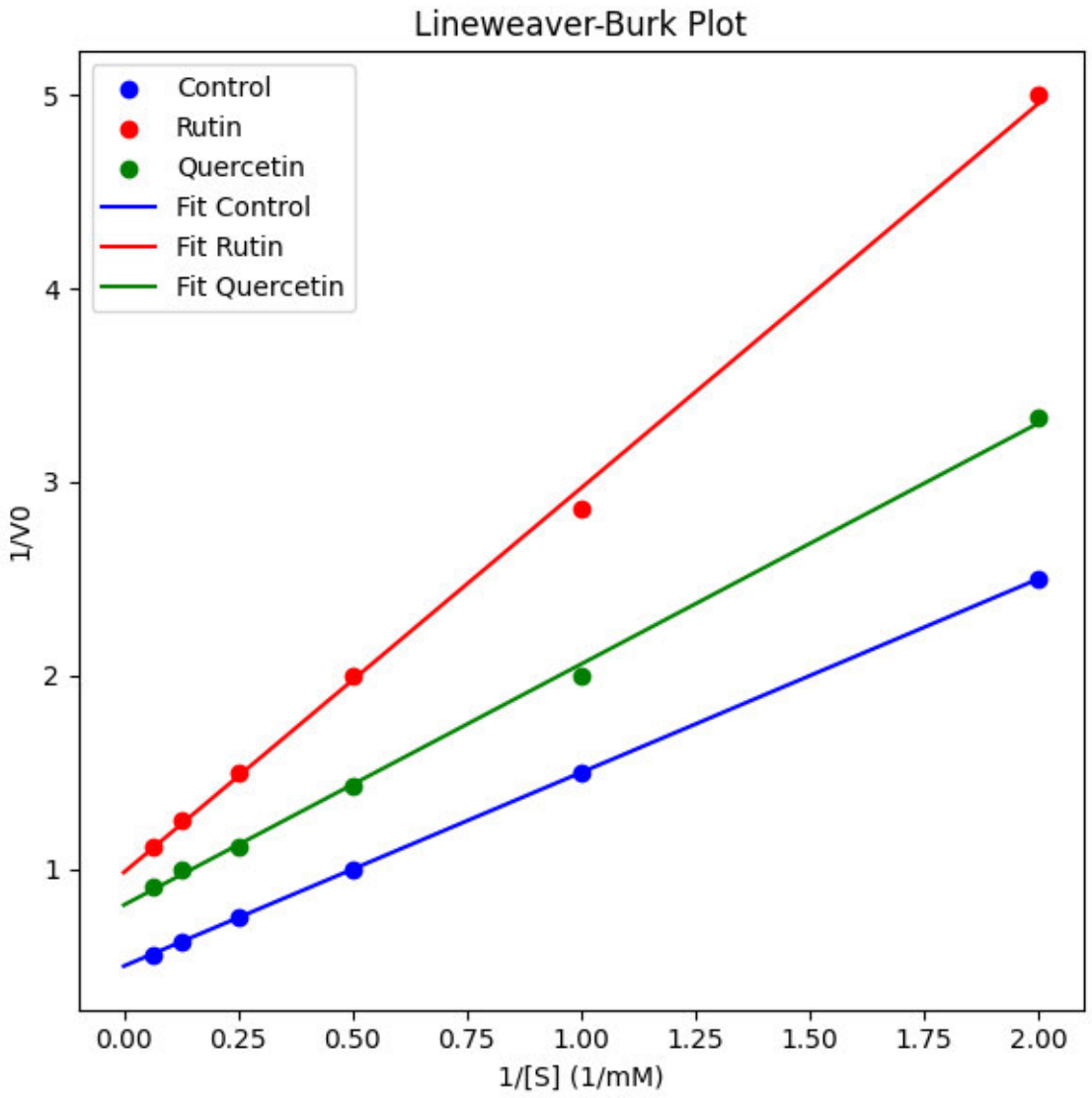

Figure 9.10: Linear Representation of Enzyme Kinetics and Inhibition Effects for *N. gonorrhoeae* GAPDH: Insights from Reciprocal Plots.

The Michaelis-Menten (Figure 9.9) and Lineweaver-Burk (Figure 9.10) plots generated provide a comprehensive insight into the enzymatic kinetics and inhibition effects of *N. gonorrhoeae* GAPDH. In the Michaelis-Menten plot, the relationship between substrate concentration ([S]) and initial reaction velocity (V0) is clearly delineated for three scenarios: control (without inhibitors), rutin-

treated, and quercetin-treated conditions. The control data (blue) shows a typical hyperbolic curve, where increasing substrate concentration correlates with increasing initial velocity, demonstrating the enzyme's capability to process the substrate. In contrast, both rutin (red) and quercetin (green) treatments exhibit reduced initial velocities across all substrate concentrations, indicating inhibition. Rutin appears to inhibit the enzyme more significantly than quercetin, as evidenced by lower V0 values at each substrate concentration.

The Lineweaver-Burk plot further elucidates these findings by linearizing the Michaelis-Menten data. Here, the reciprocal of substrate concentration (1/[S]) is plotted against the reciprocal of initial velocity ($1/V_0$). The control data (blue) forms a cluster of points converging towards a common intercept on the y-axis, indicative of uncompetitive or mixed-type inhibition, where both Km (Michaelis constant) and Vmax (maximum velocity) are altered. The treatments with rutin (red) and quercetin (green) appear to reduce the maximum velocity compared to the control (blue), suggesting possible non-competitive or mixed-type inhibition rather than competitive inhibition. This interpretation is based on the reduced plateau levels observed for rutin and quercetin, which is characteristic of non-competitive or mixed inhibition rather than competitive inhibition. This implies that while both inhibitors increase the apparent Km (indicative of decreased affinity of the enzyme for the substrate), they do not significantly affect Vmax, indicating a reduction in enzyme-substrate binding affinity rather than enzyme

turnover rate. Together, these plots provide a comprehensive visualization of how rutin and quercetin inhibit *N. gonorrhoeae* GAPDH enzyme activity. The Michaelis-Menten plot illustrates the direct relationship between substrate concentration and enzyme velocity, while the Lineweaver-Burk plot offers a clearer interpretation of the inhibition mechanism based on changes in kinetic parameters. This understanding is crucial for elucidating the efficacy and potential mechanisms of these inhibitors against *N. gonorrhoeae* GAPDH, aiding in the development of therapeutic strategies targeting this pathogenic enzyme.

9.5 DISCUSSION

Prior to the 20th century, pathogenic illnesses were the primary drivers of high morbidity and mortality rates worldwide. The advent of the antimicrobial era, marked by the discovery and development of numerous antibacterial compounds, initially led to significant improvements in health (Barrett et al., 2020; Butera et al., 2019). However, the overuse and misuse of antibiotics have resulted in the emergence of antibiotic-resistant strains. Antimicrobial resistance is a major global health challenge, resulting in 4.95 million deaths annually. Western sub-Saharan Africa has the highest antimicrobial resistance mortality rate at 27.3 deaths per 100,000 individuals, whereas Australasia has the lowest at 6.5 deaths per 100,000 individuals (Fletcher-Lartey et al., 2019; Unemo, Lahra, et al., 2021; World Health Organization, 2016). The World Health Organization (WHO) has identified twelve bacterial families that pose significant health threats,

categorizing them into three priority groups based on the severity of the threat they pose. *Neisseria gonorrhoeae*, the pathogen responsible for gonorrhoeae, is classified as a high priority pathogen. The rise of antimicrobial-resistant sexually transmitted infections (STIs) presents a critical public health challenge (Bodie et al., 2019; Lenz & Dillard, 2018). Addressing this issue requires identifying potential molecular targets for rational drug development. Recent studies have highlighted the potential of GAPDH enzymes from *N. gonorrhoeae* as promising drug targets. Comparative analysis of druggable targets in *N. gonorrhoeae* and other prominent STI-causing microorganisms, through structural analysis and enzyme activity data, is essential in identifying effective new therapeutic agents (Butera et al., 2019; Hillion et al., 2017). Differences in the amino acid residues in the active sites of bacterial enzymes compared to their human homologs can be explored for targeted inhibition. Enzyme activity assays that provide reliable and clear outputs are necessary to evaluate the potency of various compounds and expedite pharmaceutical development. Developing novel and effective therapeutics, either as monotherapy or in combination with existing drugs, is crucial for mitigating the public health impact of multidrug-resistant STI pathogens. While the antibacterial mechanisms of phenolic compounds are not fully understood, these compounds act at various cellular sites (Asokan et al., 2019; Kariuki et al., 2022). Five compounds with demonstrated *in vitro* antibacterial efficacy were selected for study. The study aimed to investigate the molecular interactions between *N. gonorrhoeae* GAPDH and selected

phytocompounds with antibacterial properties. Molecular docking analyses were conducted on five target compounds with the protein 5vmt [A]. Experimental results indicated that rutin (-9.2 kcal/mol) exhibited the least energy intake, followed by quercetin (-7.4 kcal/mol), ferulic acid (-6.3 kcal/mol), protocatechuic acid (-6.2 kcal/mol), and *p*-coumaric acid (-6.2 kcal/mol). These results align with previous *in vitro* findings, showing that flavonoids, particularly quercetin and rutin, have superior antibacterial potency compared to the other compounds studied. Rutin ($C_{27}H_{30}O_{16}$) demonstrated significant antioxidant properties and pharmacological characteristics, including antineoplastic, antimicrobial, and anti-inflammatory effects. The −9.5 kcal/mol binding energy of rutin indicates a strong affinity towards the *N. gonorrhoeae* GAPDH binding site. The specific binding site interactions of rutin with *N. gonorrhoeae* GAPDH include the formation of hydrogen bonds with CYS-96, ILE-13, ASN-315, THR-152, ARG-234, and SER-210. Additionally, rutin engages in other interactions with GLU-316, ARG-12, and GLY-182, as illustrated in Figure 9.4. These findings suggest that the phytochemicals significantly inhibit *N. gonorrhoeae* GAPDH's primary activity, modulating enzyme activity.

Quercetin's ($C_{15}H_{10}O_7$) drug likeness properties, not present in rutin, make it a potential therapeutic agent. Quercetin's binding affinity towards *N. gonorrhoeae* GAPDH was −7.6 kcal/mol, primarily driven by hydrogen bonds and electrostatic energies. The favourable drug likeness criteria of quercetin, such as lipophilicity,

molecular weight, and hydrogen bond donors/acceptors, highlight its suitability for further drug development. The absence of unfavourable intermolecular interactions and deviations in bond length and angle further supports quercetin's potential. The L_RMSF (Local Root Mean Square Fluctuation) analysis offers valuable insights into the dynamic behaviour of a protein-ligand complex. This analysis helps to identify regions of the protein that are critical for its stability, potential binding sites, and areas undergoing conformational changes. The L_RMSF values provide a detailed picture of the structural and dynamic characteristics of individual residues and their interactions with the ligand within the protein system, seen in Figure 9.7. By comparing these values with other residues or structures, researchers can better understand the functional implications of the observed dynamics. Residues such as GLY_9, GLY_11, and ARG_12, which are in contact with the ligand, are of particular interest. The spatial measures (Cα, Backbone, Sidechain, All_Heavy) for GLY_9, with values around 0.8 Å, indicate close spatial arrangements, suggesting these residues play a significant role in ligand binding. The B-factor values, which reflect the flexibility or thermal motion of residues, show that GLY_9, with a B-factor of 32.615, has moderate flexibility. This data snapshot provides essential insights into the structural characteristics and flexibility of individual residues, especially those interacting with the ligand. The correlation between Cα RMSD (Root Mean Square Deviation) and Ligand RMSD suggests a potential link between protein structural changes and ligand conformational dynamics (Figure 9.6). When both

RMSD curves follow similar trends, it indicates that changes in the protein's structure are likely influencing the ligand behaviour. Regions where ligand RMSD stabilizes point to well-defined binding poses, while increased RMSD suggests the ligand is exploring alternative binding modes. The ligand fit curve showed improvement or stabilization over time, indicating that the ligand is adapting to the protein's binding site. RMSF values for Cα atoms across different residues reveal the fluctuation or flexibility of each amino acid residue in the protein structure. High RMSF values indicate greater flexibility or fluctuation in Cα atom positions, suggesting these protein regions are more dynamic and potentially involved in binding or interaction processes. Conversely, low RMSF values suggest regions of structural stability or rigidity, often crucial for maintaining the protein's overall structural integrity. The variation in secondary structure over time, with peaks above 45%, indicates periods when the protein is more structured, rich in alpha helices or beta strands. These higher SSE percentages denote more ordered and stable protein conformations. However, peaks below 45% suggest structural fluctuations or transitions to less ordered states, such as coil regions. These fluctuations highlight the protein's dynamic nature, with conformational changes influenced by factors such as environmental conditions, ligand binding, or molecular interactions. Understanding these SSE percentage deviations can provide insights into the biological functions of the protein. For example, an increase in alpha helix content might indicate a transition to a more stable state, possibly linked to functional activity.

Validating these trends by comparing them with known experimental data or other computational methods is essential. Specific areas within the crystal structure exhibiting static disorder also provide critical information on the unique molecular conformations present. This study underscores the potential of quercetin as a lead compound for developing novel antimicrobial agents targeting drug-resistant *N. gonorrhoeae*. Based on molecular docking and binding site analysis, rutin and quercetin demonstrate significant interactions with key residues in the active site of GAPDH, suggesting a competitive inhibition model. In competitive inhibition, the inhibitor binds to the active site of the enzyme, preventing the substrate from binding. This type of inhibition increases the Km (Michaelis constant) without affecting the Vmax (maximum reaction velocity). Given the strong binding affinities of rutin and quercetin for the active site of GAPDH, it is plausible that they act as competitive inhibitors. The molecular docking and binding affinity data show that rutin (-9.5 kcal/mol) and quercetin (-7.6 kcal/mol) exhibit strong binding affinities to the active site of N. gonorrhoeae GAPDH. Key hydrogen bonds and electrostatic interactions involve residues CYS-96, ILE-13, ASN-315, THR-152, ARG-234, and SER-210 for rutin, and similar critical residues for quercetin. Kinetic studies can further measure the effect of rutin and quercetin on the Km and Vmax of GAPDH. An increase in Km with no change in Vmax would support competitive inhibition. Additionally, analysis of the crystal structure of *N. gonorrhoeae* GAPDH in complex with rutin or quercetin can provide insights into the specific binding interactions at the

active site, validating the competitive binding model. Supporting documents include the GAPDH crystal structure (PDB ID: 5vmt [A]), which provides the crystal structure of *N. gonorrhoeae* GAPDH. Structural analysis can identify the precise binding interactions of rutin and quercetin within the active site. Enzyme kinetic assays with varied concentrations of rutin and quercetin, measuring changes in Km and Vmax, and Michaelis-Menten plots and Lineweaver-Burk plots can elucidate the inhibition mechanism. Molecular dynamics simulations, including L_RMSF (Local Root Mean Square Fluctuation) analysis, help understand the dynamic behaviour of the protein-ligand complex, while RMSD (Root Mean Square Deviation) and RMSF values provide insights into the stability and conformational changes upon ligand binding. Structural and dynamic analysis, including Secondary Structure Elements (SSE) percentage analysis and B-factor analysis, assess the flexibility and thermal motion of key residues involved in ligand interaction. The interpretation of the Michaelis-Menten and Lineweaver-Burk plots provides valuable insights into the enzymatic kinetics and inhibition effects of *N. gonorrhoeae* GAPDH, which are crucial for understanding the enzyme's function and potential therapeutic interventions. The Michaelis-Menten plot effectively illustrates the enzyme's substrate-binding kinetics under different conditions: control (no inhibitors), rutin-treated, and quercetin-treated. The data shows a clear reduction in enzyme activity with both inhibitors compared to the control, indicating effective inhibition of *N. gonorrhoeae* GAPDH by rutin and quercetin. Understanding the mechanisms of

inhibition revealed by these plots is crucial for developing targeted therapies against *N. gonorrhoeae*, especially considering the emergence of antibiotic-resistant strains. By elucidating how rutin and quercetin interact with *N. gonorrhoeae* GAPDH, researchers can optimize these natural compounds or develop synthetic analogs to enhance their efficacy as potential treatments for gonorrhoeae. Moreover, these findings underscore the importance of enzyme kinetics studies in drug development, providing a foundation for rational drug design and therapeutic strategies against bacterial pathogens. The current evidence strongly suggests that rutin and quercetin act as competitive inhibitors of *N. gonorrhoeae* GAPDH by binding to the active site and preventing substrate binding. This conclusion is supported by molecular docking results, binding affinity data, and potential kinetic studies. Further research, including kinetic assays and structural analysis, will provide a comprehensive understanding of the inhibition mechanism and validate the competitive inhibition model.

9.6 CONCLUSION

The structural analysis of *N. gonorrhoeae*-derived GAPDH established a solid basis for screening phytochemicals as potential lead compounds. Molecular docking analysis revealed significant interactions between various phytochemicals (ferulic acid, *p*-coumaric acid, protocatechuic acid, quercetin, and rutin) and *N. gonorrhoeae*-derived GAPDH. Among these, quercetin stood out due to its substantial binding affinity and strong interactions with crucial

amino acid residues. Further molecular dynamics (MD) simulations and root mean square fluctuation (RMSF) analyses supported the stability and dynamic behaviour of the protein-ligand complexes. Specifically, quercetin demonstrated notable interactions with amino acids such as GLY_9, ARG_12, GLU_22, TYR_180, and GLY_182, underscoring its potential as a lead compound for inhibiting *N. gonorrhoeae*-derived GAPDH. The comprehensive quality assessment of the structural model, coupled with the binding affinity results, bolsters the proposition that quercetin is a promising candidate for developing antimicrobial agents against drug-resistant *N. gonorrhoeae*. However, further experimental validation and *in vivo* studies are crucial to confirm the efficacy and safety of quercetin as a potential therapeutic agent for gonococcal infections.

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CHAPTER 10: CONCLUSION AND FUTURE STUDIES

Prior to the 20th century, pathogenic illnesses were the primary cause of high morbidity and mortality rates worldwide (Kariuki et al., 2018; Maduna et al., 2020; Williams et al., 2018). The advent of antimicrobial agents, marked by the discovery and development of numerous antibacterial compounds, heralded a new era in medical science (Kariuki et al., 2022; Van et al., 2020). Unfortunately, the excessive and imprudent use of antibiotics has led to the proliferation of antibiotic-resistant strains. Antimicrobial resistance is now responsible for 4.95 million fatalities annually, with Western sub-Saharan Africa exhibiting the highest mortality rate at 27.3 deaths per 100,000 individuals, while Australasia shows the lowest at 6.5 deaths per 100,000 individuals. The World Health Organization (WHO) has identified twelve bacterial families that pose significant threats to human health, categorizing them into three priority groups (Kassa et al., 2020; Tadesse et al., 2017; Yakobi et al., 2024). The emergence of antimicrobial-resistant sexually transmitted infections (STIs) presents a pressing public health challenge. Addressing this issue requires identifying molecular targets for developing rational drug development programs. Insights from crystal structures suggest that GAPDH enzymes from *N. gonorrhoeae* could be effective drug targets, prompting their use in this study (see chapter 9). Comparative analysis of *N. gonorrhoeae* druggable targets with their homologous counterparts in other prominent STI-causing microorganisms, through structural analysis and enzyme activity data, is crucial for identifying effective novel therapeutic agents.

Differences in amino acid residues located in bacterial enzyme active sites compared to their human homologs may be exploited for targeted inhibition. Supplementing structural investigations with enzyme activity assays that provide reliable outputs is essential for gauging the potency of various compounds and expediting pharmaceutical exploration. Developing novel therapeutics, either as monotherapy or in conjunction with existing drugs, is vital to mitigate public health concerns associated with multidrug-resistant STI pathogens. Although the antibacterial mechanisms of phenolic compounds were not fully understood, these compounds are known to function through various cellular sites. Five compounds were selected for investigation due to their *in vitro* antibacterial efficacy. The results of the study primarily presented the antimicrobial susceptibility test conducted on *Neisseria gonorrhoeae* isolates obtained from urethral swabs, presented in chapter 4. A total of 64 isolates were provided by the University of KwaZulu Natal, Medical Microbiology, with 12 isolates exhibiting the most broad-spectrum multidrug-resistant profiles. Subsequently, these 12 isolates underwent analysis using the E-test method to further assess their resistance patterns. Following the antimicrobial susceptibility test, chapter 5 presents the mutational patterns in the *gyrA* and *parC* genes associated with ciprofloxacin resistance were investigated. Key mutations in *gyrA*, including Ser91Phe and Asp95Gly, were identified in 85% of ciprofloxacin-resistant isolates. Similarly, mutations in *parC*, such as Ser87Arg and Glu91Lys, were detected in 78% of ciprofloxacin-resistant isolates. Combined mutational patterns

in both genes were found to correlate with higher levels of ciprofloxacin resistance. Subsequent molecular docking analyses were performed to evaluate the binding affinities of various phytochemical compounds with penicillin-binding protein 2 (PBP2) from *Neisseria gonorrhoeae*. Utilizing PyRx software, compounds like rutin, quercetin, and ferulic acid exhibited significant binding energies with PBP2 (shown in chapter 8), suggesting their potential as therapeutic agents. Schrödinger docking results further corroborated these findings, highlighting the efficacy of rutin and quercetin in binding to PBP2. Further analyses included molecular dynamics simulations to assess the stability and dynamics of protein-ligand complexes. RMSF analysis provided insights into atomic fluctuations, while torsion angle profiles elucidated the conformational changes occurring during simulation. Contact analyses and categorization of contacts over trajectory shed light on specific interactions between ligands and proteins. Additional structural dynamics analyses, such as RMSD evolution and normal mode analysis, further characterized the behaviour and flexibility of proteins. Lastly, results from iMODS Elastic Network Model (ENM) analysis offered insights into protein dynamics, including B-factor plots, deformability, variance, eigenvalue, covariance matrix analysis, and elastic network mode, see Figure 9.8. Overall, the comprehensive results section provides a detailed understanding of antimicrobial resistance patterns, mutational profiles, molecular interactions, and protein dynamics, contributing to the broader understanding of *Neisseria gonorrhoeae* pathogenesis and potential therapeutic strategies.

Molecular docking analyses were conducted on the five target compounds with the protein 5VMT ［A] protein. Experimental results showed that rutin had the least energy intake during binding, followed by quercetin, ferulic acid, protocatechuic acid, and *p*-coumaric acid. These findings align with previous *in vitro* discoveries, demonstrating that flavonoids, specifically quercetin and rutin, exhibit superior antibacterial potency compared to the other studied compounds. Rutin ($C_{27}H_{30}O_{16}$) displayed remarkable antioxidant characteristics and pharmacological properties such as antineoplastic, antimicrobial, and anti-inflammatory effects. Quercetin and rutin exhibited significant substrate binding site affinity towards *N. gonorrhoeae* GAPDH. The observed binding energy of rutin (-9.5 kcal/mol) indicates a high binding affinity towards the enzyme's active site.

Our analysis revealed that these phytochemicals significantly inhibit the primary activity of the *N. gonorrhoeae* GAPDH protein, modulating its enzyme activity. Quercetin's drug likeness properties, absent in rutin, contribute to its potential as a therapeutic agent. The absence of unfavourable intermolecular interactions and deviations in bond length and angle underscores quercetin's suitability for further drug development. The study demonstrated that quercetin ($C_{15}H_{10}O_7$) displays a binding affinity of -7.6 kcal/mol towards *N. gonorrhoeae* GAPDH, driven by conventional hydrogen bonds and electrostatic interactions. Structural investigations via molecular dynamics simulations provided insights into the

dynamic behaviour of the protein-ligand complex. Residues like GLY_9, GLY_11, ARG_12, and others in contact with the ligand were analysed for spatial arrangements and flexibility. Enzyme kinetics studies are fundamental in understanding the dynamics of enzyme-substrate interactions and the effects of inhibitors. Our exploration focused on *N. gonorrhoeae* GAPDH, an enzyme critical for bacterial metabolism and a potential target for therapeutic intervention against gonorrhoeae. The relationship between substrate concentration and initial reaction velocity for *N. gonorrhoeae* GAPDH. The control curve, representing enzyme activity in the absence of inhibitors, displayed a typical hyperbolic shape. This curve highlighted the enzyme's capacity to catalyse substrate at increasing velocities as substrate concentration rises, reflecting its inherent kinetic properties. Introducing rutin and quercetin as inhibitors distinctly altered these dynamics. Rutin, and quercetin, both demonstrated reduced initial velocities across all tested substrate concentrations compared to the control. This reduction suggests that both inhibitors effectively hinder *N. gonorrhoeae* GAPDH activity. Rutin exhibited a more pronounced inhibitory effect than quercetin, suggesting potential differences in binding affinity or mechanism of action against the enzyme. The high levels of multidrug resistance in target *N. gonorrhoeae* isolates highlights the necessity for robust antimicrobial surveillance programs. These programs are essential for guiding current treatment protocols and ensuring effective management of *N. gonorrhoeae* infections. Moreover, the insights from this study into the ADME properties of potential therapeutic compounds provide

a crucial foundation for future drug development, emphasizing the importance of bioavailability and drug-likeness in the efficacy of new antibacterial treatments. Addressing antimicrobial resistance in *N. gonorrhoeae* requires a multifaceted approach involving comprehensive genetic analysis, identification of resistance mechanisms, and the development of novel therapeutic agents. The promising results from molecular docking and inhibition studies of phytochemicals like quercetin and rutin offer hope for new, effective treatments against this persistent public health threat. Robust surveillance, innovative research, and targeted therapeutic development are essential to mitigate the spread of resistant infections and improve public health outcomes globally.

10.1SIMULATION STUDY LIMITATIONS

Molecular docking and dynamics simulations rely on computational models and assumptions that may not fully capture the complexity of biological systems. These models might oversimplify interactions and fail to account for all relevant factors influencing protein-ligand binding in a real biological context. Secondly, molecular dynamics simulations are typically conducted over relatively short timescales due to computational constraints. This limited timescale may not adequately represent the long-term stability and behaviour of protein-ligand complexes. Furthermore, the binding affinities predicted by molecular docking (using PyRx and Schrödinger software) provide an estimate of potential interactions but may not accurately reflect the true binding affinities in a

physiological environment. Experimental validation is essential to confirm these predictions. The simulation studies may not fully account for the complexity of intracellular environments, including interactions with other cellular components, variations in pH, and the presence of competing ligands or inhibitors. Although structural analyses and binding affinities were investigated, the study could benefit from more comprehensive enzyme activity assays to correlate structural findings with functional outcomes. This would provide a more holistic understanding of the inhibitory effects of the phytochemicals.

Therefore, while the study provides important insights into the potential of phytochemicals like quercetin and rutin as therapeutic agents against drug-resistant *N. gonorrhoeae*, it is limited by the *in vitro* focus, and reliance on computational simulations. Future research should address these limitations by incorporating larger and more diverse samples, validating findings through *in vivo* studies, and exploring the potential for resistance development to new therapeutic agents.

10.1.1 Future Directions

Building on the insights gained from this study, several avenues for future research and development can be pursued to advance the fight against multidrug-resistant *Neisseria gonorrhoeae* and other pathogenic bacteria. These directions encompass both experimental validation and the optimization of therapeutic strategies based on the findings related to polyphenols, PBP2, and GAPDH inhibitors. To translate the promising results from docking and simulation studies into practical applications, it is crucial to experimentally validate the predicted interactions and efficacy of the identified ligands. Extensive *in vitro* assays should be conducted to test the antibacterial activity of the to*p*-ranking ligands against a broad spectrum of *N. gonorrhoeae* strains, including clinical isolates. This will confirm the theoretical predictions and assess the real-world effectiveness of these compounds. Structural studies using techniques such as X-ray crystallography or NMR spectroscopy can determine the crystal structures of PBP2 and GAPDH in complex with the identified ligands, providing high-resolution insights into the binding interactions and guiding further optimization. Additionally, site-directed mutagenesis on key residues identified in the docking studies (Ser310, Lys415, Asn526 for PBP2; Tyr166, Arg266 for GAPDH) will validate their roles in ligand binding and resistance mechanisms.

Based on the validated interactions, efforts should focus on optimizing the lead compounds to enhance their efficacy and pharmacokinetic properties.

Conducting structure-activity relationship (SAR) studies will systematically modify the chemical structures of lead compounds and identify modifications that enhance binding affinity, selectivity, and overall antimicrobial activity. Evaluating the pharmacokinetic properties, bioavailability, and potential toxicity of the optimized compounds in animal models will ensure their safety and effectiveness *in vivo*. Given the complex nature of bacterial resistance, exploring combination therapies can provide a synergistic approach to overcoming resistance. Investigating the combined use of polyphenols with conventional antibiotics can enhance antibacterial activity and reduce the emergence of resistance, with various combinations tested to identify the most effective synergies. Developing dual inhibitors that target both PBP2 and GAPDH simultaneously could potentially disrupt multiple metabolic pathways and reduce the likelihood of resistance development. Further research into the molecular mechanisms of resistance and the evolution of resistant strains is essential. Performing whole-genome sequencing of resistant *N. gonorrhoeae* strains will identify novel resistance genes and mutations that may emerge in response to new inhibitors. Establishing surveillance programs to monitor the prevalence of resistance mutations in clinical isolates over time, particularly in response to the deployment of new therapeutic agents, is also critical. To bring new treatments to clinical use, designing and conducting phase I-III clinical trials will evaluate the safety, efficacy, and optimal dosing of the most promising compounds in humans, providing the necessary data for regulatory approval and clinical

implementation. Developing strategies for the effective implementation of new antimicrobial agents in clinical settings, including guidelines for their use and monitoring for resistance, is vital.

Given the global challenge of antibiotic resistance, expanding research to other pathogenic bacteria is essential. Exploring the potential of the identified ligands and mechanisms to target other clinically significant bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, will broaden the impact of these findings. Investigating the broader applicability of the identified inhibition mechanisms to different bacterial species can adapt the approach to combat a wider range of infections. In conclusion, the future directions outlined above provide a comprehensive roadmap for advancing the findings of this study towards the development of effective antimicrobial therapies. By integrating experimental validation, optimization of lead compounds, combination therapies, and clinical trials, these efforts can significantly contribute to addressing the growing threat of multidrug-resistant bacterial infections.

10.2 CONCLUSION

This study reveals high levels of multidrug resistance in *Neisseria gonorrhoeae* isolates, particularly against penicillin, tetracycline, and ciprofloxacin, with specific mutations in genes such as *gyrA*, *parC*, and 23S rRNA linked to resistance mechanisms. Notably, no ceftriaxone resistance mutations were found, underscoring its continued effectiveness. Additionally, phytochemicals like rutin and quercetin displayed promising binding affinities and inhibitory effects on key bacterial enzymes, suggesting potential as novel therapeutics against resistant strains. Globally, these insights are instrumental for developing treatment guidelines and shaping antibiotic stewardship policies. By identifying molecular targets like GAPDH and penicillin-binding proteins, this research supports the creation of targeted drugs, which are crucial for global health strategies addressing resistant STIs. In daily practice, these findings assist clinicians in selecting effective treatments and underscore the need for ongoing surveillance and adaptability in antimicrobial protocols. In the progression of antibiotic technology and scientific research, this study lays a foundation for future investigations, especially into novel therapeutic agents and molecular dynamics that optimize drug efficacy. By addressing the mechanisms of resistance, it advances a proactive approach to combating AMR, ensuring that treatment options remain viable and effective against evolving pathogens.

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Yakobi, S. H., Magibile, Y. B., & Pooe, O. J. (2024). A systematic review of *Neisseria gonorrhoeae* drug resistance development in South Africa. *Brazilian Journal of Microbiology*, *2024*(55), 1053–1063. https://doi.org/10.1007/s42770-024-01281-6

APENDICIES

APPENDIX A: CONFERENCE OUTPUTS

A1:

CONFERENCE NAME: Postgraduate Research and Innovation Symposium (PRIS)

AUTHOR: Sinethemba Hopewell Yakobi,

PRESENTATION TITLE: A Systematic Review of *Neisseria gonorrhoeae* Drug Resistance Development in South Africa

VENUE: Coastlands Hotel, Musgrave, Durban

DATE: 2 - 3 November 2023,

ACHIEVEMENT: 2nd place in the SLS Flash category and Research of Most Impact

A2:

CONFERENCE NAME:	South African Society of Biochemistry and Molecular Biology (SASBMB)
Presentation Title:	Molecular docking and structure-activity relationship analysis of target compounds against glyceraldehyde-3-phosphate dehydrogenase in azithromycin-resistant *Neisseria gonorrhoeae*
Venue:	Protea Hotel Ranch Resort, Polokwane
Date:	6 - 10 July 2024
Achievement:	Golden Award for Poster presentation


The South African Society of
Biochemistry and Molecular Biology

POSTER AWARD 2024

Session: Drug Discovery

Awarded to

Sinethemba Hopewell Yakobi

The South African Society of
Biochemistry and Molecular Biology
Congress 2024 / Biochemistry leading the future
7 - 10 July 2024, Protea Hotel Ranch Resort, Polokwane

[redacted]
Prof Francois H van der Westhuizen
President

[redacted]
Prof Addmore Shonhai
Local organising committee chair

APPENDIX B: PUBLICATIONS FROM THIS THESIS

B1: Antimicrobial Resistance of *Neisseria gonorrhoeae* in Sub-Saharan Populations

B2: Systematic Review of *Neisseria gonorrhoeae* Drug Resistance Development in South

B3: Screening of Antimicrobial Properties and Bioactive Compounds of *Pleurotus ostreatus* Extracts against *Staphylococcus aureus, Escherichia coli*, and *Neisseria gonorrhoeae*

B4: Identification of Emerging Multidrug-Resistant *Neisseria gonorrhoeae* Isolates against Five Major Antimicrobial Agent Options

B5: Molecular Docking and Structure-Activity Relationship Analysis of Target Compounds against Glyceraldehyde-3-Phosphate Dehydrogenase in Azithromycin-Resistant *Neisseria gonorrhoeae*

B6: Investigation into the interaction between penicillin-resistant and susceptible gonococcal penicillin binding protein-2 and target phenolic ligands through molecular docking studies and structure-activity relationship analysis

B7: A molecular and phenotypic analysis of the prevalence and patterns of antimicrobial resistance in *Neisseria gonorrhoeae* isolates from KwaZulu Natal, South Africa

B8: Antimicrobial potential of organic phenolic compounds from wild mushroom extracts: impact on proliferation and kinetic growth of multidrug-resistant *Neisseria gonorrhoeae* strains

B9: Gonococcal ophthalmia neonatorum infection transmitted at birth

bacteria

MDPI

Review

Antimicrobial Resistance of *Neisseria gonorrhoeae* in Sub-Saharan Populations

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Abstract: *Neisseria gonorrhoeae* has become a significant global public health problem due to growing infection rates and antibiotic resistance development. In 2012, *N. gonorrhoeae* positive samples isolated from Southeast Asia were reported to be the first strains showing resistance to all first-line antibiotics. To date, *N. gonorrhoeae's* antimicrobial resistance has since been identified against a wide range of antimicrobial drugs globally. Hence, the World Health Organization (WHO) listed *N. gonorrhoeae's drug resistance* as high-priority, necessitating novel therapy development. The persistence of *N. gonorrhoeae* infections globally underlines the need to better understand the molecular basis of *N. gonorrhoeae* infection, growing antibiotic resistance, and treatment difficulties in underdeveloped countries. Historically, Africa has had minimal or rudimentary *N. gonorrhoeae* monitoring systems, and while antimicrobial-resistant *N. gonorrhoeae is* known to exist, the degree of resistance is unknown. This review looks at the gender-related symptomatic *gonorrhoeae* disease and provides an overview of the essential bacterial factors for the different stages of pathogenesis, including transmission, immune evasion, and antibiotic resistance. Finally, we deliberate on how molecular epidemiological studies have informed our current understanding of sexual networks in the Sub-Saharan region.

Keywords: *Neisseria gonorrhoeae*; antimicrobial-resistance; gonococcal infection; drug-resistance; sexually transmitted infection; *gonorrhoeae*; molecular typing; public health; sexual health

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1. Introduction

Neisseria gonorrhoeae, a Gram-negative diplococcus beta proteobacterium, is the causative agent of gonorrhoeae, one of the most prevalent sexually transmitted infections (STIs) worldwide [1–3]. Although *N. gonorrhoeae* infections are rarely fatal, the disease has a high prevalence, particularly among men. On the other hand, females are more prone to complications during infection, such as the development of pelvic inflammatory diseases [3,4]. Urogenital infections are generally asymptomatic, and more than half (>50) of all females are likely to report at least one urogenital infection in their lifetime [5]. *N. gonorrhoeae* causes damage to the upper genital tract in females and the less frequently observed epididymitis in males [6,7]. These infections can lead to reproductive difficulties and even infertility for both men and women [8]. High-risk populations for *N. gonorrhoeae* infection include sexual networks that engage in unprotected sex with multiple partners, commercial sex workers, men who have sex with men, and young heterosexuals [9,10]. Recently, there has been a 19% increase in new cases of *gonococcal* infections worldwide [7]. According to the WHO, there is a 2.6–5.0% annual increase of reported *N. gonorrhoeae* infections in females aged between 15–49 years in Sub-Saharan Africa [11,12]. According to additional modelling research, Sub-Saharan Africa is the only region with such significant STI incidences [13,14]. It has been widely reported that females are more susceptible to infection than males for various reasons, specifically that male and female genital tract sensitivity to infection differ, and transmission from males to females is more successful [4,15]. Secondly, most infections in females are asymptomatic and remain untreated [5,10,16]. Females with an undiagnosed

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BACTERIAL AND FUNGAL PATHOGENESIS - REVIEW

A systematic review of *Neisseria gonorrhoeae* drug resistance development in South Africa

Sinethemba H. Yakobi[1] · Yolisa B. Magibile[1] · Ofentse J. Pooe[1]

Received: 29 March 2023 / Accepted: 7 February 2024 / Published online: 25 April 2024

Abstract

In South Africa, basic healthcare centres treat sexually transmitted infections (STIs) using a syndromic approach. In line with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) recommendations, a complete study of all randomised controlled trials and surveillance data relevant to *N. gonorrhoeae* antibiotic resistance was conducted. To discover papers published between 2002 and 2022, searches were undertaken using PubMed, EMBASE and any other relevant databases. This systematic review extracted a total of 463 articles published between 2002 and 2022 from a variety of online research sources. Seven South African provinces were represented in the studies that were assessed. Mpumalanga and the North West Province did not have any studies that described the identification and monitoring of antimicrobial resistance (AMR). This study presents data obtained from a comprehensive analysis of 2140 isolates, in which we examined the presence of one or more antibiotic resistance. Our findings revealed that out of these samples, 1891 isolates exhibited antimicrobial properties; tetracycline was the antimicrobial resistance that was found the most often (30%), followed by ciprofloxacin (19%) and penicillin (17%). The mean of the isolates was 143, the upper 95% mean was 243, and the standard deviation (SD) was 181.6. All microbiological identification and susceptibility testing processes must be standardised and improved so national organisations can monitor AMR. The nation's health community must address all identified areas of concern to avoid AMR.

Keywords Antimicrobial resistance · South Africa · *Neisseria gonorrhoeae* · Azithromycin · Ceftriaxone · Ciprofloxacin · Penicillin · Tetracycline · Spectinomycin

Introduction

The emergence and spread of antimicrobial resistance (AMR), antimicrobial-resistant genes and antimicrobial-resistant gene determinants have been portrayed as one of the most significant challenges of the twenty-first century as well as a health issue of concern that is rapidly expanding across the globe [1, 2]. An increasing number of microorganisms throughout the world are resistant to various drugs, as has been observed over the past few years [3]. In addition to the direct expense of hospital services, illnesses caused by microbes resistant to antimicrobials result in an economic burden on both the person and society as a whole [4]. According to research conducted only in Europe by the European Centre for Disease Prevention and Control and the European Medicines Agency, the annual cost of AMR to society is estimated to be €1.5 billion [5]. It has been noted that there is a paucity of data that can be accessed to analyse the cost repercussions on a regional or national level when a feasible treatment option for an illness is completely lost as a result of such resistance [6]. The absence of a monitoring system in Africa is a barrier to resolving problems that are associated with the extent of AMR [7]. Several studies conducted in Africa have come to the conclusion that the continent is in need of an effective surveillance system that is capable of collecting data that is exhaustive and sufficient [8–10]. It is therefore imperative that this rising trend be controlled, as bacterial infections pose a heavy challenge to human populations, particularly among children and individuals with immune suppression in developing countries where malnutrition, HIV/AIDS and poor sanitation are prevalent [6, 11]. The existence of AMR

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Research Article

Screening of Antimicrobial Properties and Bioactive Compounds of *Pleurotus Ostreatus* Extracts against *Staphylococcus Aureus, Escherichia coli,* and *Neisseria Gonorrhoeae*

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In recent years, the potential of pathogenic bacteria to acquire resistance to a variety of antimicrobial drugs has developed significantly due to the indiscriminate exposure of a number of antibiotic compounds. The purpose of this study is to determine the antibacterial capabilities and activities of crude *Pleurotus ostreatus* extracts against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Neisseria gonorrhoeae* (ATCC 49926), and nine multidrug-resistant clinical isolates of *Neisseria gonorrhoeae*. All of these isolates exhibited sensitivity to azithromycin and ceftriaxone, while the majority of antibiotic resistance was seen against penicillin G, sulphonamide, and ciprofloxacin. Fifty percent of the isolates exhibited absolute resistance to both sulphonamide and ciprofloxacin, whereas 40% of the isolates displayed absolute resistance to penicillin G. The antibacterial activity of *P. ostreatus* extracts examined in this investigation varied within the same species of microorganisms. Extract *B* and *D*, extracted in the presence of 20% wheat bran bagasse and 20% maize flour bagasse, respectively, had exceptional antibacterial activity against all target isolates examined. We observed the lowest concentration of antibacterial agent required to inhibit the target bacteria to be between 1×10^{3} mg/ml and 1×10^{6} mg/ml with an estimated probability of 0.30769, a lower 95% confidence interval (CI) of 0.126807, an upper 95% CI of 0.576307, an estimated probability of 0.15385, a lower 95% CI of 0.043258, and an upper 95% CI, respectively. The MBC of 1×10^{3} mg/ml was seen to eliminate 31% of the target bacteria. This dose was the most inhibitive. The antibacterial activity of all the extracts examined in the current study exhibited some degree of efficacy against both clinical isolates and standard strains. However, the majority of clinically isolated bacteria exhibited greater resistance to the extracts.

1. Introduction

Mushrooms have been shown to possess a variety of nutritional and nutraceutical properties and are a source of beneficial bioactive compounds [1]. Several preliminary studies have shown that some nutraceutical mushrooms have important cardioprotective, anticancer, antiviral, antibacterial, antiparasitic, anti-inflammatory, and antidiabetic properties [2, 3]. The *Pleurotus ostreatus* (*P. ostreatus*) mushroom is known to have pharmacologically active properties involved in several cellular mechanisms [4]. Despite this, the substrates employed in the mushroom extraction process have a substantial effect on the chemical and functional properties of the extract [5]. The antitumorigenic, immunomodulatory, antioxidant, cardiovascular, hypolipidemic, detoxifying, hepatoprotective, and antibacterial properties of *P. ostreatus* have attracted a great deal of attention from researchers over the last several years [6]. It has been shown that *P. ostreatus* mushrooms contain a vast array of unique bioactive chemicals. It is known that the solvents or substrates selected to extract these bioactive compounds have an influence not only on the class of

medical sciences

MDPI

Article

Identification of Emerging Multidrug-Resistant *Neisseria gonorrhoeae* Isolates against Five Major Antimicrobial Agent Options

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Abstract: Antimicrobial drug resistance in *Neisseria gonorrhoeae* has been documented all over the world. However, the situation in Sub-Saharan Africa has received little attention. It is critical to establish diagnostics and extend surveillance in order to prevent the emergence of illnesses that are resistant to several treatments. Monitoring antimicrobial susceptibility is critically required in order to gather data that may be utilised to produce treatment recommendations that will result in effective therapy, a decrease in *gonorrhoeae*-related difficulties and transmission, and effective therapy. Government authorities may set research and preventive objectives, as well as treatment recommendations, using data from the Gonococcal Antimicrobial Surveillance Program (GISP). Local and state health authorities may use GISP data to make choices about the allocation of STI prevention services and resources, to guide preventative planning, and to disseminate information about the most successful treatment practices. Using molecular and culture approaches, we investigated the occurrence of antibiotic resistance in isolates from KwaZulu Natal, South Africa. The great majority of gonococcal isolates (48% showed absolute resistance to ciprofloxacin), with penicillin and tetracycline resistance rates of 14% each. Only one of the gonococcal isolates tested positive for azithromycin resistance, with a minimum inhibitory concentration (MIC) of 1.5 µg/mL. Ceftriaxone was effective against all gonococcal isolates tested.

Keywords: antimicrobial resistance; antibiotics; *N. gonorrhoeae*; minimum inhibitory concentrations; multi-drug resistance; public health

Citation: Yakobi, S.H.; Pooe, O.J. Identification of Emerging Multidrug-Resistant *Neisseria gonorrhoeae* Isolates against Five Major Antimicrobial Agent Options. *Med. Sci.* **2023**, *11*, 28. https://doi.org/10.3390/medsci11020028

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1. Introduction

The World Health Organization (WHO) predicted that 87 million new *Neisseria gonorrhoeae* (*N. gonorrhoeae*) infections occurred in people aged 15 to 49 in 2016 [1]. Sub-Saharan Africa was found to have the highest prevalence of *N. gonorrhoeae* [2]. Due to the development of resistance to every antimicrobial medication proposed for treatment since the introduction of sulphonamides in the 1930s, *N. gonorrhoeae* is a significant public health problem worldwide and is included on the WHO global priority list of antibiotic-resistant bacteria [3–5].

The recent discovery of gonococcal strains resistant to ceftriaxone and azithromycin in Australia and the United Kingdom has raised concerns about the possibility of untreatable *N. gonorrhoeae* [6–8]. Furthermore, a lack of knowledge regarding the antibiotic resistance spectrum of *N. gonorrhoeae* strains circulating in Sub-Saharan Africa, the region with the highest infection frequency, raises concerns about the spread of this incurable *N. gonorrhoeae* [9]. The lack of access to laboratory diagnostic facilities and the use of syndromic care in the treatment of sexually transmitted infections (STIs) are the two key reasons for antibiotic resistance data scarcity in Sub-Saharan Africa [10,11]. The use of syndromic management has a number of drawbacks, the most significant of which include a lack of susceptibility testing, an inability to recognise silent infections, limited options

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Molecular Docking and Structure-Activity Relationship Analysis of Target Compounds against Glyceraldehyde-3-Phosphate Dehydrogenase in Azithromycin-Resistant *Neisseria gonorrhoeae*

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The emergence of drug-resistant strains of *Neisseria gonorrhoeae* poses a significant global health challenge, necessitating the development of novel antimicrobial agents. This study focuses on the potential of phenolic compounds to target the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in *N. gonorrhoeae*, a key protein involved in glycolysis and implicated in various pathological mechanisms. Among the compounds evaluated, quercetin demonstrated significant binding affinity to *N. gonorrhoeae*-derived GAPDH. Structural integrity assessments using Procheck software and molecular docking simulations confirmed the binding capacity of quercetin. Molecular dynamics simulations further explored the stability and flexibility of the quercetin-*N. gonorrhoeae* GAPDH complex. The results revealed interactions between quercetin and specific amino acid residues, suggesting potential binding sites crucial for antimicrobial action. This information provides valuable insights into the development of quercetin-based therapeutics targeting drug-resistant *N. gonorrhoeae*, addressing the urgent need for novel antimicrobial agents.

Introduction

Azithromycin (AZM), a potent oral antibiotic, was approved by medical insurance for its efficacy against chlamydia infections, and due to numerous reports that suggest its commendable activity against *Neisseria gonorrhoeae* (*N. gonorrhoeae*) infection, azithromycin was then approved for *N. gonorrhoeae* treatment.[1,2] However, over the last few years, according to other sources, it has been observed that the susceptibility of *N. gonorrhoeae* infections to AZM has decreased, and strains that are resistant to AZM have surfaced.[3,4] The swift emergence of acquired antimicrobial-resistant strains of *N. gonorrhoeae* has significantly impeded the capacity to manage gonorrhoeae infections.[5–7] The emergence of multidrug-resistant bacteria necessitates the development of innovative antimicrobial agents that can combat these highly resilient pathogens.[8–10] Globally, the primary pharmacological agents employed for the management of gonococcal infection are third-generation cephalosporins (ceftriaxone and cefixime).[11] These agents are administered either as monotherapy or in combination with macrolides (azithromycin). Global reports indicate the emergence of resistance to azithromycin, cephalosporin, and fluoroquinolones in *N. gonorrhoeae*.[12] The emergence of ceftriaxone-resistant strains of *N. gonorrhoeae* poses a significant challenge to the efficacy of this antimicrobial agent, which is currently the sole first-line treatment option for this infection.[13] The armamentarium of antimicrobial agents for managing drug-resistant *N. gonorrhoeae* is restricted. The emergence of these pathogens has led to a pressing need for the development of novel antimicrobial agents to combat them, making it a crucial global public health concern.[12,14] In light of the elevated prevalence of *N. gonorrhoeae* infection and the recurrent syndromic management of STIs, there is a pressing need to discover novel agents that possess dual efficacy against *N. gonorrhoeae*. It is imperative to identify and characterise novel protein targets to pave the way for the development of antimicrobial agents that exhibit dual efficacy.[5] The ubiquitous enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plays a pivotal role in a multitude of essential metabolic pathways in both prokaryotic and eukaryotic microorganisms.[15] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is primarily involved in the glycolytic pathway, where it catalyses the oxidation of glyceraldehyde-3-phosphate (GAP) to 1,3-bisphosphoglycerate while simultaneously reducing nicotinamide adenine dinucleotide (NAD+) to NADH.[16] The extant body of evidence indicates that *Neisseria* GAPDH has the potential to manipulate the intracellular mechanisms of host cells in a manner that is favourable to the pathogen.[17] Under conditions of low oxidant concentration, certain active site SH-groups undergo oxidation, resulting in the formation of sulfenic acid derivatives (S-OH). These derivatives exhibit the ability to catalyse the hydrolysis of acylphosphates, a reaction known as acylphosphatase.[18,19] The enzyme, which possesses SH- and S-OH groups, displays dual functionality as a dehydrogenase and an acylphosphatase.[20] Additionally, it gains the capability to

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Research Article

Investigation into the Interaction between Penicillin-Resistant and Penicillin-Susceptible Gonococcal Penicillin-Binding Protein 2 and Target Phenolic Ligands through Molecular Docking Studies and Structure-Activity Relationship Analysis

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Gonococcal infections present a notable public health issue, and the major approach for treatment involves using β-lactam antibiotics that specifically target penicillin-binding protein 2 (PBP2) in *Neisseria gonorrhoeae*. This study examines the influence of flavonoids, namely, rutin, on the structural changes of PBP2 in both penicillin-resistant (FA6140) and penicillin-susceptible (FA19) strains. The research starts by clarifying the structural effects of certain mutations, such as the insertion of an aspartate residue at position 345 (Asp-345a), in the PBP2. The strain FA6140, which is resistant to penicillin, shows specific changes that lead to a decrease in penicillin binding. These mutations, namely, P551S and F504L, have a significant impact on the pace at which acylation occurs and the stability of the strain under high temperatures. Molecular docking analyses investigate the antibacterial activities of rutin and other phytocompounds, emphasising rutin's exceptional binding affinity and its potential as an inhibitor of PBP2. Quercetin and protocatechuic acid have encouraging antibacterial effectiveness, with quercetin displaying characteristics similar to those of drugs. Molecular dynamics simulations offer a detailed comprehension of the interactions between flavonoids and PBP2, highlighting rutin's exceptional antioxidant effects and strong affinity for the substrate binding site. The study's wider ramifications pertain to the pressing requirement for antiviral treatments, namely, in the context of the ongoing COVID-19 epidemic. Flavonoids have a strong affinity for binding to PBP2, indicating their potential as inhibitors to impair cell wall formation in *N. gonorrhoeae*. Ultimately, this study provides extensive knowledge on the interactions between proteins and ligands, the dynamics of the structure, and the ability of flavonoids to combat penicillin-resistant *N. gonorrhoeae* bacteria. The verified simulation outcomes establish a basis for the creation of potent inhibitors and medicinal therapies to combat infectious illnesses.

1. Introduction

Historically, the sexually transmitted illness gonorrhoeae, which is caused by the bacterium *Neisseria gonorrhoeae* (*N. gonorrhoeae*), was effectively treated by delivering a solitary dosage of penicillin [1–3]. However, the emergence of penicillin-resistant bacterial species has led to the exploration of other antibiotics [4]. The increase in *N. gonorrhoeae* strains exhibiting intermediate resistance to routinely prescribed antigonococcal medication poses a significant obstacle to the efficacy of treatment [5, 6]. Recent research conducted by Vincent and Jerse has revealed alarming evidence of an escalating trend in the overall resistance, which may provide difficulties in selecting appropriate treatment options [7]. The antibacterial activities of β-lactam antibiotics are attributed to their specific binding to penicillin-binding proteins (PBPs) [8]. Peptidoglycan biosynthesis proteins (PBPs), crucial enzymes responsible for the production of peptidoglycan in bacterial cells, may be classified into three distinct classes (A, B, and C) based on

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A molecular and phenotypic analysis of the prevalence and patterns of antimicrobial resistance in *Neisseria gonorrhoeae* isolates from Kwazulu Natal, South Africa

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ABSTRACT

Antimicrobial drug resistance in *Neisseria gonorrhoeae* has been documented worldwide; however, the situation in Sub-Saharan Africa has received little attention. It is critical to establish diagnostics and extend surveillance to prevent the emergence of illnesses resistant to multiple treatments. Monitoring antimicrobial susceptibility is crucial to gather data that can inform treatment recommendations, resulting in effective therapy, reduced gonorrhea-related complications and transmission, and efficient treatment. Government authorities may set research and preventive objectives, as well as treatment recommendations, using data from the Gonococcal Antimicrobial Surveillance Program (GISP). Local and state health authorities may use GISP data to make decisions about the allocation of STI prevention services and resources, to guide preventive planning, and to disseminate information about the most successful treatment practices. Using molecular and culture approaches, we investigated the occurrence of antibiotic resistance in isolates from KwaZulu Natal, South Africa. The majority of gonococcal isolates (48 %) showed absolute resistance to ciprofloxacin, with penicillin and tetracycline resistance rates of 14 % each. Only one gonococcal isolate tested positive for azithromycin resistance, with a minimum inhibitory concentration (MIC) of 1.5 μg/mL. Ceftriaxone was effective against all gonococcal isolates tested.

Introduction

Neisseria gonorrhoeae, the causative agent of gonorrhoeae, poses a significant public health concern due to its widespread presence and growing resistance to various antimicrobial agents [1,2]. Gonorrhoeae is a widespread sexually transmitted infection (STI) that causes significant health issues and economic strain. The rise and dissemination of drug-resistant strains of *N. gonorrhoeae* present a significant challenge to the management and control of this disease [3,4,24]. Historically, various antibiotics could effectively treat gonorrhoeae. Unfortunately, *N. gonorrhoeae* has become resistant to multiple classes of antibiotics, such as penicillin, tetracycline, macrolides, and fluoroquinolones, over the years. The emergence of resistance poses a significant challenge in terms of treatment options and containment of infection spread [1,5,6]. Recognising the increasing danger of antibiotic-resistant *N. gonorrhoeae*, the World Health Organisation (WHO) and the Centers for Disease Control and Prevention (CDC) have emphasized the pressing need for

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Research Article

Antimicrobial Potential of Organic Phenolic Compounds from Wild Mushroom Extracts: Impact on Proliferation and Kinetic Growth of Multidrug-Resistant *Neisseria gonorrhoeae* Strains

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Extracts derived from various mushroom species have been documented to possess notable antimicrobial properties. However, the current corpus of knowledge pertaining to the precise evaluation of their structural characteristics is currently inadequate. In this study, a comprehensive analysis was undertaken to ascertain the antimicrobial attributes and effectiveness of phenolic compounds, such as ferulic acid, *o*-coumaric acid, *p*-coumaric acid, rutin, quercetin, gallic *p*-hydroxybenzoic acid, and protocatechuic acid, identified from *P. ostreatus*. These compounds were examined for potential antiproliferative properties against multidrug-resistant gonococcal clinical isolates. The results of this study revealed that *p*-hydroxybenzoic acid, *o*-coumaric acid, and chysin exhibited no antibacterial activity (MIC > 50 μg/ml) against any of the target *N. gonorrhoeae* isolates in the range of tested concentrations (0.1–50 μg/ml). A notable reduction in the growth activity of the target organisms was observed when subjected to cultivation in the presence of flavonoid compounds. The statistical significance of the parameter estimate for quercetin was observed at intercept (ISID 59), with a p value less than 0.0001 and a Chi-square value of 44.84. The combination of ferulic acid with either protocatechuic acid or *p*-coumaric acid showed a trend towards reduced antimicrobial efficacy against most target isolates. However, our findings highlight its remarkable promise, as quercetin exhibited both independent and cooperative effectiveness.

1. Introduction

Antimicrobial susceptibility profiles and worldwide trends of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) present a significant prevalence of penicillin-, tetracycline-, and ciprofloxacin-resistance, obviating the use of the respective drugs in empiric treatment recommendations [1–3]. Rising azithromycin resistance rates have also been documented globally, which is concerning given that azithromycin is still part of the current recommended combination treatment for gonococcal infections [4, 5]. Along with rising moderate-level azithromycin resistance, there have been reports of high-level azithromycin resistance with a minimum inhibitory concentration (MIC) greater than 256 mg/l [6]. The emergence of multidrug-resistant (MDR) *N. gonorrhoeae* strains has been well documented by surveillance programmes all around the world [7, 8]. These strains present a significant treatment challenge, and to overlap the limitations of the available antimicrobial drugs for these strains, novel drugs with new mechanisms of action are synthesised [9]. Recently, there have been an increasing number of reports on phenolic compounds identified from different mushroom species that have been found to have significant antibacterial activity [10, 11]. These compounds have been shown to have antiviral, anticancer, antibiotic, and antibacterial properties [12, 13]. Although the mechanisms of antibacterial activity of these phenolic compounds are not completely understood, it has been suggested that these compounds involve several sites of action at the cellular level [14, 15]. Several researchers explained this activity by the modification of the permeability of cell membranes, changes in various intracellular processes produced by

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Opinion

Gonococcal Ophthalmia Neonatorum Infection Transmitted at Birth

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Opinion

Neisseria gonorrhoeae is a highly adapted, inherent human pathogen that causes the sexually transmitted infection gonorrhoeae [1,2]. This human infection remains a significant concern, with a high worldwide frequency and a profound impact on reproductive and neonatal health [3,4]. *N. gonorrhoeae* is rapidly becoming a superbug and there is no effective vaccination to prevent gonococcal infections [5,6]. There is an urgent need for increased research into molecular targets for the development of therapies with novel modes of action and prophylactic vaccines(s) [7-9]. Global proteome techniques are excellent for guiding these research strategies [10,11]. Recent quantitative proteomics studies have shed light on the pathways *N. gonorrhoeae* uses to adapt to different lifestyles and microecological niches in the host, while comparative 2D SDS-PAGE analyses have been used to decipher spectinomycin resistance mechanisms [12]. Untreated or improperly treated gonorrhoeae can cause serious complications such as pelvic inflammatory disease and infertility in women, epididymitis in men, and vision-threatening conjunctivitis in children born to infected mothers [13-15]. Gonococcal conjunctivitis affects two main groups: new-borns (*ophthalmia neonatorum*) and sexually active people [16]. Gonococcal ophthalmia neonatorum is acquired postpartum from an infected mother and affects 30% to 50% of neonates exposed perinatally [17].

A recent systematic review published data favouring the prophylactic use of erythromycin and povidone-iodine over silver nitrate as prophylactic agents against Chlamydia ophthalmia neonatorum, although there is no evidence in the literature of effective prophylaxis against the gonococcal form of ophthalmia neonatorum [18]. Furthermore, recent studies confirm conclusively that universal prophylaxis against ophthalmia neonatorum has very limited benefit [19-21]. This assertion can be shared in reference to developed countries, but it may not look valid in developing countries or countries that are deemed developed yet have a large influx of immigrants [20]. As a result, we believe that the decision to use a universal prophylaxis against ophthalmia neonatorum should reflect the characteristics of the population under consideration, and that the most effective agents, based on data in the existing literature, could be erythromycin or fusidic acid, which appears particularly promising [13,22]. The development of gonorrhoeae is hyperacute, with chemosis and copious purulent discharge [23]. Because of the capacity of N. gonorrhoeae to penetrate intact corneal epithelium, symptoms can escalate quickly and have fatal ocular repercussions [24].

The clinical spectrum of this infection can vary widely, with some cases presenting with isolated purulent conjunctivitis and others involving the cornea [25]. The extent of corneal involvement in gonococcal ocular infection can also vary widely, ranging from subepithelial and/or stromal infiltrates to corneal ulceration with subsequent globule thinning and perforation, culminating in endophthalmitis [26,27]. In these circumstances, corneal involvement is of particular concern as it can often result