Formulation of nanostructured lipid carriers using eugenol and D-α-tocopherol succinate for the inhibition of bacterial efflux pumps to enhance delivery of ciprofloxacin.

By

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“A winner is a dreamer who never gives up.”

-Nelson Mandela-

"***This dissertation is dedicated to my grandmother, siblings, friends, andteachers for their unconditional love and unwavering support.***

I hope I made you proud."

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Research output from the dissertation

Abstract

Background: The continuously growing antibacterial resistance crisis is decreasing the availability of the antibiotics to treat bacterial infections. Bacteria develop additional mechanisms to survive lethal concentrations of antibiotics such as efflux pumps and biofilms. Novel drug delivery systems are urgently required to enhance antibiotic efficacy and overcome resistance. Furthermore, natural derivatives from medicinal plants have shown great potential as bacterial adjuvants to inhibit these mechanisms. Therefore, employing these compounds in the formulation of nanocarriers could restore antibiotic efficacy and overcome antibiotic resistance.

Aim: The aim of this study was to explore the potential of ciprofloxacin-loaded nanostructured lipid carriers (CIP-NLCs) designed using D-α-tocopherol succinate (TS) and eugenol for enhancing antimicrobial activity and overcoming resistance mechanisms against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (*P. aeruginosa*).

Methods: CIP-NLCs were prepared using hot homogenization/ultrasonication method. The particle size, polydispersity index (PDI) and zeta potential (ZP) of CIP-NLCs were determined using the dynamic light scattering technique. Transmission electron microscopy analysis was conducted to confirm particle size and visualize the morphology of CIP-NLCs. The entrapment efficiency (EE %) of CIP-NLCs was determined using the ultrafiltration method and was quantified using High-Performance Liquid Chromatography (HPLC). *In vitro* drug release of CIP-NLCs were conducted using the dialysis bag technique and CIP released was quantified using HPLC. Drug release kinetics were analysed using the DDSolver program. Haemocompatibility of CIP-NLCs was performed using sheep blood. The in vitro antibacterial activity of CIP-NLCs were determined using micro broth assay against *Staphylococcus aureus* (SA), Escherichia *coli* (*E. coli),* MRSA, and *P. aeruginosa*. Bacterial killing kinetics were performed against MRSA and *P. aeruginosa* using the plate colony counting method. MRSA and *P. aeruginosa* biofilm inhibition of CIP-NLCs was evaluated using microtiter method. DPPH scavenging was used to study the antioxidant activity of CIP-NLCs. *In vivo* antibacterial activity of MRSA was studied using the systemic MRSA infection on BALB/c mice.

Results: CIP-NLCs had a particle size, PDI, ZP, and EE % of 147.4 ± 0.59 nm, 0.219 ± 0.009, -9.64 ± 2.22 mV, and 82.8 ± 0.39 %, respectively. The *in vitro* biosafety evaluation revealed CIP-NLCs as non-haemolytic. The *in vitro* drug release study showed a biphasic release of CIP from the CIP-LNCs for 48 hours at physiological pH (7.4). The *in vitro* antibacterial activity of CIP-NLCs (SA:0.195 µg/mL and MRSA:12.5 µg/mL) showed 2-fold lower minimum inhibitory concentration (MIC) values over bare ciprofloxacin (SA: 0.39 µg/mL and MRSA: 25 µg/mL) against MRSA, SA, whereas the MIC values of CIP-NLCs (*E. coli*: 0.048 µg/mL and *P. aeruginosa*: 0.097 µg/mL) were 4-fold lower CIP (*E. coli*: 0.195 µg/mL and *P. aeruginosa*: 0.39 µg/mL) against *E. coli*, and *P. aeruginosa.* The bacterial-killing kinetic test showed 100% elimination of MRSA and *P. aeruginosa* within eight and

one hour(s) of treatment with CIP-NLCs, respectively. Conversely, 100% elimination of MRSA and *P. aeruginosa* was shown within 24 and 12 hours of treatment with bare ciprofloxacin, respectively. CIP-NLCs eliminated 3-fold MRSA biofilm compared to bare ciprofloxacin, whereas 1.25-fold *P. aeruginosa* biofilms were eliminated. The efflux pump inhibition potential of CIP-NLCs was confirmed using cartwheel assay, which showed high fluorescence intensity on bacteria treated with CIP-NLCs. The DPPH scavenging assay of CIP-NLCs proved antioxidant activity equivalent to Vitamin C (ascorbic acid), which is reported to be a potent antioxidant. The *in vivo* systematic infection in BALB/c mice reduced MRSA infection in kidney, liver, and blood by 12.27-fold, 4.47-fold, and 1613-fold, respectively.

Conclusion: CIP-NLCs designed using TS and eugenol could enhance the antimicrobial activity against MRSA and *P. aeruginosa* infections and inhibit efflux pumps associated with these bacteria. Therefore, the CIP-NLCs may serve as a promising tool for enhanced delivery of ciprofloxacin and treatment of bacterial infections.

Keywords: Ciprofloxacin-loaded nanostructured lipid carriers D-α-tocopherol succinate; efflux pumps; bacterial infections.

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Tables of abbreviations

ATP	Adenosine triphosphate	NDDS	Nano drug delivery system
ABS	Absorbance	*P. aeruginosa*	Pseudomonas aeruginosa
CIP	Ciprofloxacin	PBS	Phosphate buffer saline
DLS	Dynamic light scattering	PDI	Polydispersity index
DNA	Deoxyribose nucleic acid	PEG	PEGylated
DPPH	2,2-diphenyl-1-picrylhydrazyl	PIT	Phase inversion temperature
E. coli	Escherichia coli	PLNs	
EE %	Entrapment efficiency	PS	Particle size
EPS	Extra polymeric substance	o/w	Oil in water
EtBr	Ethidium bromide	RND	Resistance-nodulation-cell-division superfamily
FIC	Fractional inhibitory concentration	QRDR	Quinolone resistance determining region
HPH	High-pressure homogenization	SA	Staphylococcus aureus
HPLC	High-Performance Liquid Chromatography	SADD	Self-assembled drug delivery system
LBNs	Lipid based nanoparticles	SD	Standard deviation
MIC	Minimum inhibitory concentration	SLNs	Solid lipid nanoparticles
MFS	Major facilitator superfamily	TEM	Transmission electronic microscopy
MHA	Muller Hinton agar	TS	D-α-tocopherol succinate
MHB	Muller Hinton broth	UV	Ultraviolet
MRSA	Methicillin-resistant Staphylococcus aureus	VCM	Vancomycin
n	Mean	w/o	Water in oil

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Chapter One
Introduction

1.1 Introduction

This chapter introduces the study with a brief background, outlining the current status of infectious diseases due to bacterial infections, limitations of current antibiotic therapy, and the considerably growing crisis of antibacterial resistance. Moreover, this chapter applies efflux pump inhibition to improve nano antibiotic delivery systems to address multidrug resistant diseases and biofilm formation. This is followed by the aims, objectives, novelty, and significance of the study and concludes with a brief overall structure and contents of the thesis.

1.2 Background

Infectious diseases are among the top ten leading contributors to the significantly increasing rate of mortality and morbidity [1, 2]. The World Health Organization (WHO) 2018 report claimed 17 million global fatalities as attributable to infectious diseases. Among these infectious diseases, bacterial infections pose a considerable threat as a significant cause of death. It is estimated that 7.7 million deaths worldwide in 2019 were due to bacterial infections [3,4]. Consequently, bacterial infections are regarded as the primary cause of death to all infectious diseases[1]. Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (*P. aeruginosa*) have a rapidly growing resistance profile causing mild illnesses such as endocarditis, skin infections, osteomyelitis, and life-threatening diseasessuch as sepsis and pneumonia [6]. In 2019, the recent systematic review in the Lancet reported 100,000 global fatalities as caused by MRSA infections [5]. Conversely, the Centres for Disease Control and Preventionpredicted that in 2017, *P. aeruginosa* caused 2,700 fatalities, globally [7]. Therefore, novel therapeutic strategiesto overcome this burden are urgently required.

In recent decades, wide-spectrum antibiotics have been successfully used to treat bacterial infection. However, the growing emergence of resistant bacteria has contributed to the ineffectiveness of several antibiotics, limiting their therapy to bacterial infections. Antibiotic resistance is defined as the phenomenon where bacteria develop tolerance towards bactericidal and inhibitory effects of different antibiotics [8]. Fatalities from antibiotic resistance are estimated to rise to 10 million in 2050 [9]. The widespread challenge of antibiotic resistance is primarily caused by globalization, poor sanitation, and indiscriminate use of antibiotics. Consequently, these factors contribute to the continually increasing resistance mechanisms as they lead to genetic variations and mutations of the bacteria. The common strategies that induce bacterial antimicrobial resistance include enzymatic destruction of the drug, drugtarget site modification, and lowering intracellular drug concentration by either the active efflux pumpsor changes in membrane permeability [10, 11], drug modification, and drug suppression **(Figure 1)** [12].

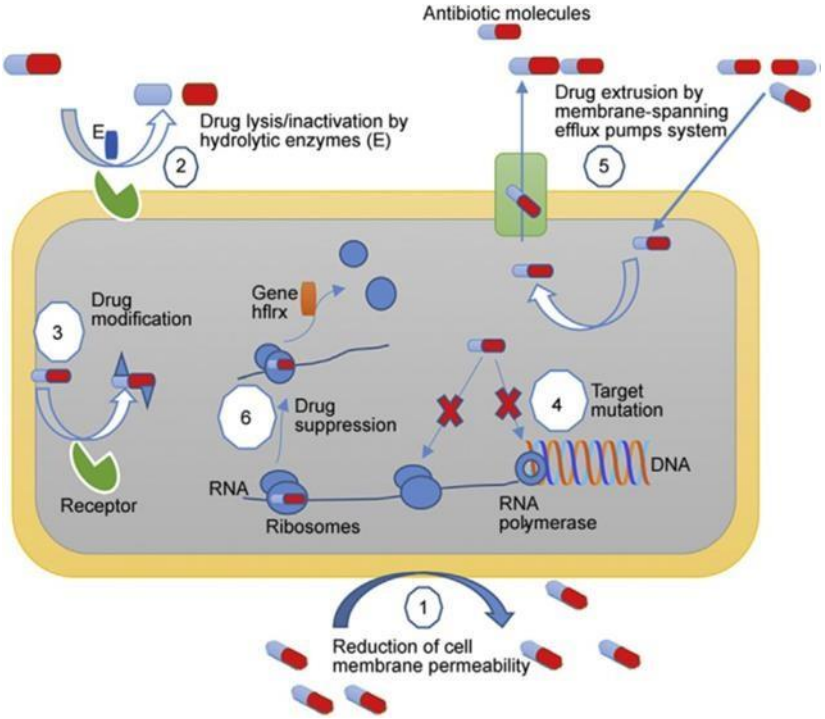

Figure 1: Different mechanisms of bacterial resistance [13].

Overexpression of bacterial efflux pumps is a substantial global concern as they are reported to be the primary component for antibiotic resistance. These trans-proteins are expressed on chromosomes and plasmids of bacteria [14], and they extrude a broad range of substrates, including fluoroquinolones, tetracycline, biocidal, monovalent cation, and dyes like ethidium bromide and acridine orange [15]. Additionally, efflux pumps cause mutation at the binding sites of antibiotics, as the bacteria are exposed to subinhibitory concentrations of the drugs [16]. The classes of efflux pumps, such as major facilitator superfamily (MFS) and resistance-nodulation-cell-division (RND) superfamily, particularly *NorA* and *NorB*, *AcrA*, and *MexA* have been extensively researched in *Staphylococcus aureus* (SA) and *P. aeruginosa*, respectively [17]. In addition to the antibiotic challenge, over 60% of bacterial infections result from biofilms. Biofilms are a community of matrix-enclosed bacterial cells adherent to inert or biological surfaces that produce extracellular polymeric matrix (EPS) [18]. The EPS provides protection to sessile bacteria and their phenotypical differences renders biofilms 10-1000 times more resistant to antibiotics in comparison to planktonic bacterial cells [19], and their eradication requires increased antibiotic concentrations [20, 21].

The advent of antibiotic therapies in the 1940s was a significant milestone that revolutionized infection prevention, control, and treatment outcomes, extending life expectancy [22]. Since their initial discovery, conventional dosage forms such as tablets, creams, capsules, suspension, and so on have been used to administer antibiotics. However, the current dosage forms of antibiotics are associated with subtherapeutic concentrations at the bacterial infection sites due to poor pharmacokinetics profiles, resulting in poor penetration, low bioavailability, and short circulation time[23]. Larger doses are required to achieve the anticipated therapeutic outcomes, and that has the potential to maximize

the adverse side effects of the drug. The well-known limitations of the current antibiotic dosage and increased access without proper sanitation, resulting in irrational antibiotic use, are primary contributors to the development of the ever-increasing antibiotic resistance challenge. Therefore, there is an urgent need to optimize strategies to improve treatment for bacterial infections while overcoming the limitations of conventional dosage forms.

In this regard, nanocarriers are proposed as an ultimate solution to maximize antibiotic therapy and address the urgent antibiotic resistance crisis. Their capability to efficiently deliver different classes of antibiotics to the microenvironment, coupled with their unique physicochemical properties, make them ideal candidates for superior antibacterial therapies to counteract antibiotic resistance [24]. Moreover, antibiotics incorporated into nanocarriers show desired characteristics such as sustained drug release and selective targeting, thus improving the bioavailability at the infection site [25, 26]. Consequently, this potentially reduces the drug exposure to the healthy cells, leading to toxicity and prolonging the circulation time of the loaded drug [27]. Additionally, nanoparticles show significant advances in the treatment of biofilm infections by disrupting the biofilm formation and potentially targeting the bacterial cells protected in the EPS matrix [28]. Numerous nanocarriers have been investigated for their potential to deliver antibiotics, including solid nanoparticles [29], dendrimers [30], liposomes [31], micelles [32], polymeric nanoparticles [33], nanoemulsions [34], and mesoporous silica nanoparticles [35]. Regrettably, nanocarriers present bottlenecks that compromise their applicability in bacterial infection therapy and overcoming antibiotic resistance, such as low loading capacity, biocompatibility, and targeting properties [36].

To this end, different nanocarriers have been investigated for their potential to improve intracellular antibiotic concentrations by exploiting the antimicrobial resistance mechanisms and factors synonymous with bacterial infection site [37, 38]. Recently, multi-disciplinary research centres have shown tenacity in finding strategies to reverse efflux pumps and regain the therapeutic value of various antibiotics, as efflux proteins play a major role in multidrug resistance [39]. Hence, several nanoparticles have been investigated for efflux pump inhibitory effect against MRSA, including copper nanoparticles [40], supramolecular self-assembled drug delivery systems (SADD) [41], and solid lipid nanoparticles (SLNs) [42], among others. Therefore, the above studies provide a platform to broaden the research efforts towards formulating other efflux pump inhibitory nanocarriers with low toxicity concerns.

Nanostructured lipid carriers (NLCs) have recently garnered significant attention from researchers as they boast desired characteristics such as enhanced loading efficiencies, prolonged drug release [43], and their ability to load both hydrophobic and hydrophilic antimicrobial agents, cost-effectiveness, andlarge-scale production feasibility [44]. NLCs are derivatives of solid lipid nanoparticles composed of the solid lipid matrix mixed with a specified amount of liquid lipids [45]. The increased entrapment efficiency in NLCs is due to the imperfect structure of their lipid matrix attributing from solid and liquidlipids blend [46, 47]. In

addition, the structural nature of NLCs allows the use of variable lipids to target different classes of efflux pumps. Despite the unique characteristics of NLCs to all other lipid nanoparticles, no study has evaluated their ability to overcome bacterial efflux pumps. Hence, there is a need to identify the lipids possessing inherent efflux pump inhibitory activity to offer improved delivery of antibiotics to the bacterial interior.

D-α-tocopherol succinate (TS) and eugenol are proposed as the efflux pump adjuvants to be optimized for the formulation of the NLCs. Eugenol is a phenylpropanoid derived from clove oil that improves the non-specific permeability of the bacterial membrane [48]. The antimicrobial activity of this compound depends on its hydrophobicity and phenolic structure, facilitating its interaction with the target cell. Eugenol is reported to inhibit MexA and AcrA to improve the antibacterial therapy of antibiotics against *P. aeruginosa* and *Escherichia coli* (*E. coli*) [48]. Therefore, these desirable properties highlight eugenol as an ideal liquid lipid to fabricate efflux pump inhibitory NLCs. In addition, like other phenol compounds, eugenol possesses antioxidant properties that allow the maintenance of free radicals in the body. To date, no study has explored the advantages of eugenol in a nanocarrier for the delivery of antibiotics.

In addition to the above, TS is proposed as a solid lipid demonstrating intrinsic anti-efflux properties [49]. TS is a lipid-soluble isoform with many biological activities, including antimicrobial and antioxidant activities. The antimicrobial activity of this compound is increased by their ability to improve the penetration and cellular uptake of substances such as antibiotics owing to their increased lipophilicity in the bacterial cell wall. TS modifies the bacterial cell membranes enabling the permeability of substances that could result in the damage of critical elements and the proton collapse [50]. Although the efflux pump inhibitory activity of TS has been documented in two nanocarriers [42, 41], no study has formulated NLCs using TS and evaluated their efflux pump inhibitory activity.

Herein, we report efflux pump inhibitory NLCs, composed of TS and eugenol, as the lipid phase of the nanoformulation. These nanocarriers are prepared using hot ultrasonication/homogenization method to combine the lipid phase with the aqueous phase containing ciprofloxacin hydrochloride (CIP) and Tween 20 as the emulsifier. The NLCs' ability to encapsulate the hydrophilic and hydrophobic antibiotics is due to their lipid and water phases, respectively. These novel nanoparticles will be evaluated for their potential for improving the antibacterial efficacy of the loaded CIP against SA, MRSA, *P. aeruginosa, and E. coli*, reduction of associated biofilms, and antioxidant activity. This is the first study to optimize efflux pump inhibitory NLCs for any drug class. In addition, this study investigates the efflux pump inhibitory capabilities of eugenol and TS against the gram-positive and gram-negative bacteria to prevent the extrusion of substances via *in vitro* efflux pump inhibition studiespresented by the accumulation of ethidium bromide (EtBr).

1.3 Problem statement

The continuously increasing issue of antimicrobial resistance has been acknowledged as a hindrance to treating bacterial infectious diseases, leading to a global public health issue. This issue is a significant contributing factor to the limitations of the current conventional dosage forms of antibiotics leading to the subtherapeutic concentrations of antibiotics at bacterial infection sites, such as non-selective targeting, short drug circulation time, low bioavailability, and poor permeation to the infected tissues/cells. Efforts to overcome these limitations using conventional antibiotics would require frequent and higher therapeutic doses, raising the risk of dose-dependent harmful and toxic effects. Furthermore, the overexpression of efflux pumps associated with resistant bacteria such as *NorA*, *NorB*, *MexA*, and *AcrA* is highlighted as a primary cause of multidrug resistance scourge as they expel various classes of antibiotics and other substances. In addition, biofilm formation further complicates the therapy of bacterial infections as they are ten times more resistant than planktonic bacteria. This prompts innovative strategies to improve the treatment of the current antibiotic therapies against the bacterial burden. Introducing nano drug delivery systems has notably enhanced the antibiotic concentration at the infection site. However, the efflux of antibiotics and the formation of biofilms still need to be addressed to further improve the treatment of such infections with nanocarriers. Therefore, lipids reported to possess inherent efflux pump inhibitory activity may be used for the formulation of antibiotic loaded NLCs, potentially increasing antibacterial therapy and overcoming resistance mechanisms. To our knowledge, no study has formulated efflux pump inhibitory NLCs for any antibiotic.

1.4 Research question

Research Question: Can CIP loaded NLCs formulated with eugenol and tocopherol succinate exhibit improved encapsulation efficiency and demonstrate a sustained drug release profile, biocompatibility, antioxidant, antibiofilm, and anti-efflux pump activities?

1.5 Aims and objectives.

The aim of this study was to explore the potential of CIP loaded NLCs designed using TS and eugenol for the inhibition of bacterial efflux pumps and biofilms, enhanced antimicrobial activity, and overcoming resistance mechanisms against MRSA and *P. aeruginosa*.

In order to achieve this aim, the objectives of the study were:

1. To prepare CIP-loaded NLCs from tocopherol succinate and eugenol.

2. To optimize and characterize CIP-loaded NLCs in terms of size, Polydispersity index (PDI), zeta potential, entrapment, morphology, *in vitro* drug release profile.

3. To investigate *in vitro* haemolytic effect of the prepared CIP-NLCs to confirm the biocompatibility.

4.To perform *in vitro* antibacterial activity, *in vitro* anti-biofilm activities and bacterial killing kinetics.

5. To investigate *in vitro* efflux pump inhibition of CIP-NLCs.

6. To assess *in vitro* antioxidant activity for CIP-NLCs against DPPH free radicals.

7. To determine *in vivo* antibacterial efficacy of CIP-NLCs.

1.6 Novelty of the Study

This study is novel due to the following reasons:

- Although efflux pump inhibitory nanoparticles have been previously studied [41], this is the first report of efflux pump inhibitory NLCs for any class of antibiotics.
- Whilst eugenol has been encapsulated as an active in a nanocarrier system to explore its antimicrobial and anti-efflux activities [51-53], this is the first study to use eugenol as an excipient to prepare an efflux pump inhibitory nanocarrier itself.
- Efflux pump inhibitory nanoparticles have been previously formulated using TS [42]. However,this is the first study to prepare NLCs using tocopherol succinate as an excipient to inhibit effluxpumps.

1.7 Significance of the Study

This study reports that efflux pump inhibitory NLCs drug carrier designed from eugenol and TS, present a novel and promising approach for improving the drug interaction with the bacteria. It will enhance the drug concentration at the infection site and activity, thereby overcoming antibiotic resistance. The potential significance of the formulation explored in this study are:

New pharmaceutical products

This study proposes the new NLC formulation with an efflux pump inhibitory effect as a medicine for improved disease treatment. This novel pharmaceutical medicine could provide a primary platform for the pharmaceutical industry to explore efflux pump inhibitory nano drug delivery systems.

Improved treatment of bacterial infections

This novel efflux inhibitory nanocarrier will sustain drug release profiles, inhibit efflux pumps and antibacterial activity, thereby increasing drug concentration at the infection site. The other outcome of these advances would be the prevention of resistance towards various antibiotic classes.

Stimulation of new research

This study could provide new potential research directions for:

- The fabrication of different types of nanoparticles with efflux pump inhibitory activity for applying different antibiotic classes and treating other diseases.
- Further exploration of other bacterial behavioural factors will provide the potential to improve disease treatment and improve life expectancy.

1.8 Overview of the Dissertation

The research is presented in the following chapters:

Chapter 1. Introduction:

This chapter provides a brief background of the study, including the current status of bacterial infectious diseases, limitations of conventional dosage, the significantly growing scourge of antibiotic resistance, and biofilm formation. Furthermore, it provides an overview of nanocarriers and discusses the benefits of efflux pump inhibitory nanocarriers in overcoming current antibiotic and antibiotic resistance limitations. It further provides the aims and objectives, novelty, and significance of the study and concludes with a brief overall structure and content of this thesis.

Chapter 2. Literature review:

This chapter provides an overview of the current burden of infectious diseases and the well-documented limitations associated with traditional doses of antibiotic therapy, resulting in the development of bacterial resistance. It outlines the seriousness of antimicrobial resistance and elucidates how overexpression of efflux pumps significantly challenges disease treatment outcomes. Consequently, it highlights the potential nano drug delivery systems have as a novel strategy in alleviating traditional dose limitations and antimicrobial resistance. It provides a comprehensive understanding of nanostructured lipid carriers and concludes by describing ciprofloxacin as a model drug.

Chapter 3. Manuscript:

This chapter addresses the aims and objectives stated in chapter 1, and it is a first-authored experimentalarticle to be submitted to the journal for publication. This article presents optimized ciprofloxacin- loaded nanostructured lipid carriers (CIP-NLCs) with multi-functional therapy against bacterial infections. *In vitro* studies efflux pump inhibitory capabilities of CIP-NLCs against efflux pumps associated with *P. aeruginosa* and MRSA investigated. In addition, different characterizations performed on the formulation, such as *in vitro* drug release, *in vitro* biosafety, and *in vitro* antibacterialand antioxidant properties are discussed.

Chapter 4. Conclusion:

This chapter describes the overall conclusions reached in achieving the study aims, outlines the significance of the study, and makes recommendations for future research in the efflux pump inhibitory antibiotic-loaded nanocarriers.

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Chapter Two

Literature review

2.1 Introduction

This chapter provides an overview of the current burden of infectious diseases and the well-documented limitations associated with traditional doses of antibiotic therapy, resulting in the development of bacterial resistance. It outlines the seriousness of antimicrobial resistance and elucidates how overexpression of efflux pumps significantly challenges disease treatment outcomes. Consequently, it highlights the potential nano drug delivery systems have as a novel strategy in alleviating traditional dose limitations and antimicrobial resistance. It provides a comprehensive understanding of nanostructured lipid carriers and concludes by describing ciprofloxacin as a model drug.

2.2 Infectious Disease Burden and Antibacterial Drug Therapy Limitations

Infectious diseases, mainly due to bacterial infections, remain a global threat despite the substantial scientific efforts to improve their therapies [1]. Infectious diseases are associated with increased morbidity and fatalities rates worldwide [2]. Infectious diseases are reported to cause 17 million deaths, which accounts for 57% of the top ten leading causes of death [3]. Meanwhile, 7.7 million fatalities per yearin 2019 were due to bacterial infections [4], highlighting them as the primary cause of death among infectious diseases [3, 5]. The difficulties of bacterial infections range from moderate

(cutaneous) to fatal illnesses like pneumonia, tuberculosis, sepsis, and others. In a study, Farrell has reported bacterialcoinfection or secondary infections as most common in lethal cases of SARS-CoV-2 diseases [6, 7].In addition, sepsis-related deaths reported in 2017 accounted for 20% of annual global deaths, a rate higher than any other disease [8].

Of more concern, bacteria continuously acquire the ability to develop genes that enable them to counteract the actions of antimicrobial agents, which may demonstrate either bactericidal or bacteriostatic activities against them, as shown in **Figure 1** [11, 12]. Some bacterial strains possess one antibiotic-resistant gene, while others have multiple resistant genes leading to multidrug resistance. Multidrug resistance is highlighted as a critical worldwide health challenge due to untreatable strains associated with raisedmortality and morbidity, such as Mycobacterium tuberculosis [13], methicillin-resistant *Staphylococcus aureus* (MRSA) [14], and *Pseudomonas aeruginosa (P. aeruginosa)*. The World Health Organization (WHO) reported that 700,000 people die from resistant bacterial infections per year. Moreover, studies reportedthat MRSA caused more than 100,000 global deaths in 2019 [15]. The number of fatalities due to resistant bacterial infections is projected to reach 10 million per year worldwide by 2050 [16]

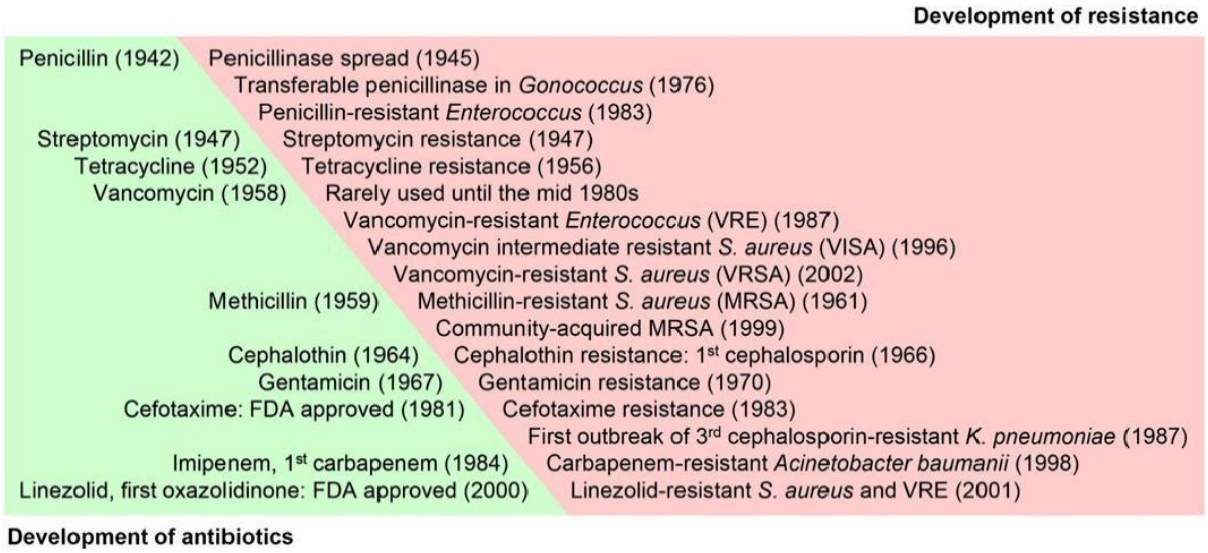


Figure 1: Development rate of new antibacterial agents and antibacterial resistance [17].

In addition to the above, the ability of bacterial strains such as MRSA and *P. aeruginosa* to develop resistance to several classes of antibiotics is not limited to only planktonic cells but also extends to biofilms. Biofilms are bacterial cells that are permanently adhesive to a surface and fixed in an extracellular polymeric substance [17]. The EPS-matrix joins the bacteria to the biofilm and forms a strong fortress against the host immune system and adverse conditions, among which are antibacterial effects. These biofilms demonstrate increased antibiotic resistance, rendering them more highly persistent than their counterparts [18]. Studies report that biofilms are associated with 80% of resistant infections in the body, including endocarditis, infection in the urinary tract, infection from indwelling medical devices, and the formation of dental plaques [19]. Due to the significantly increasing

antimicrobial resistance and evolution of microbes, systems to overcome resistance at a molecular level are of indispensable necessity.

Despite the initial profound success of antibiotics in the 1940s, effective antibacterial therapies and novel strategies are urgently needed to treat and prevent infections [20]. Currently, conventional dosage forms of antibiotics are challenged by well-reported limitations associated with sub-optimal concentrations at bacterial infection sites. These include poor tissue/cell penetration, premature drug release, low bioavailability, poor pharmacokinetic profiles, lack of targeting, and short circulation time [21, 22]. Additionally, achieving the proven therapeutic value of these antibiotics would necessitate administering large and frequent doses, thus increasing the risk of adverse effects and poor patient compliance. These limitations associated with antibiotics, along with their rampant use, are the primary contributors to the antimicrobial resistance crisis [23, 24]

2.3 Nanoengineered delivery for enhancing antibiotic therapy

In the face of the current antibiotic conventional dosage forms challenges and antimicrobial resistance, nanotechnology offers a promising strategy to improve antibiotic therapy and alleviate antimicrobial resistance. Nanotechnology is recognized as a new paradigm in designing, manufacturing, and applying nanosized materials for optimizing new therapies to treat infectious diseases [25]. Novel nanosized drug delivery systems (NDDS) are preferred in antimicrobial applications as they can combat bacterial infections and act as carriers of different classes of antibiotics and natural antimicrobial compounds [26]. Nanocarriers exhibit unique physiochemical properties, including flexible tunability in size, a large ratio of surface area to mass, desired interaction with microbes while reducing toxicity to host's cells, surface hydrophilicity and hydrophobicity, and capacity for structural and functional modification [27, 28].

2.3.1 Advantages of nanocarriers for antibiotic therapy

Nanocarriers are acknowledged as ideal delivery systems for antibiotics, holding several advantages over conventional dosage forms, including improved pharmacokinetics profiles and therapeutic index while reducing the required dose to achieve their proven clinical effects [29]. Incorporating antibiotics in nanocarriers considerably enhances targetability which minimize their interaction with the body's secretions and inhibit the production of substances the bacteria use as their defense mechanisms, elevating the active drug concentration at the site of infection [30]. These NDDS also demonstrate enhanced cellular uptake, bioavailability, biocompatibility, uniform bio-distribution, and sustained drug release [31]. These unique pharmacokinetics profiles of nanocarriers for antibiotic delivery represent superior antibacterial capabilities and reduced systemic side effects. Nanocarriers can exert their antibacterial

activity through variable mechanisms, including direct interaction with the bacterial cell wall, triggering adaptive and innate immune responses, scavenging free radicals, and inhibiting biofilm formation [32, 33]. Moreover, lipids and other excipients used in designing these nanosystems are reported to inherently demonstrate various properties with enhanced antibacterial capabilities in overcoming different drug- resistance mechanisms [34]. Further to the above, the issue of antibiotic resistance is prevented by the potential of nanocarriers to incorporate multiple antibiotics representing highly complex antimicrobial mechanisms of action [25].The increased antibiotic concentration in the microenvironment is attributed to the capability of nanocarriers to improve membrane penetration and prevent the degradation and efflux of the active dose [35]. Interestingly, nanosystems, due to their small sizes, can efficiently penetrate through the tinypores of the EPS matrix on the biofilm structure and eradicate sessile bacteria [36].

There is a vast repertoire of organic and inorganic compounds for antimicrobial therapies providing various advantages to antibiotics (displayed in **Figure 2**), which includes solid lipid nanoparticles (SLNs) [37], liposomes [38], dendrimers [39], micelles [40], polymeric nanoparticles [41], nanoemulsions[42], and mesoporous silica nanoparticles [43]. Organic nanosystems are preferred to inorganic nanosystems due to their bioactivity, easy molecular structural modification, and reduced toxicity [44]. Therefore, the appropriate design and application of various nanoparticles will present tremendous therapeutic outcomes for antibiotics.

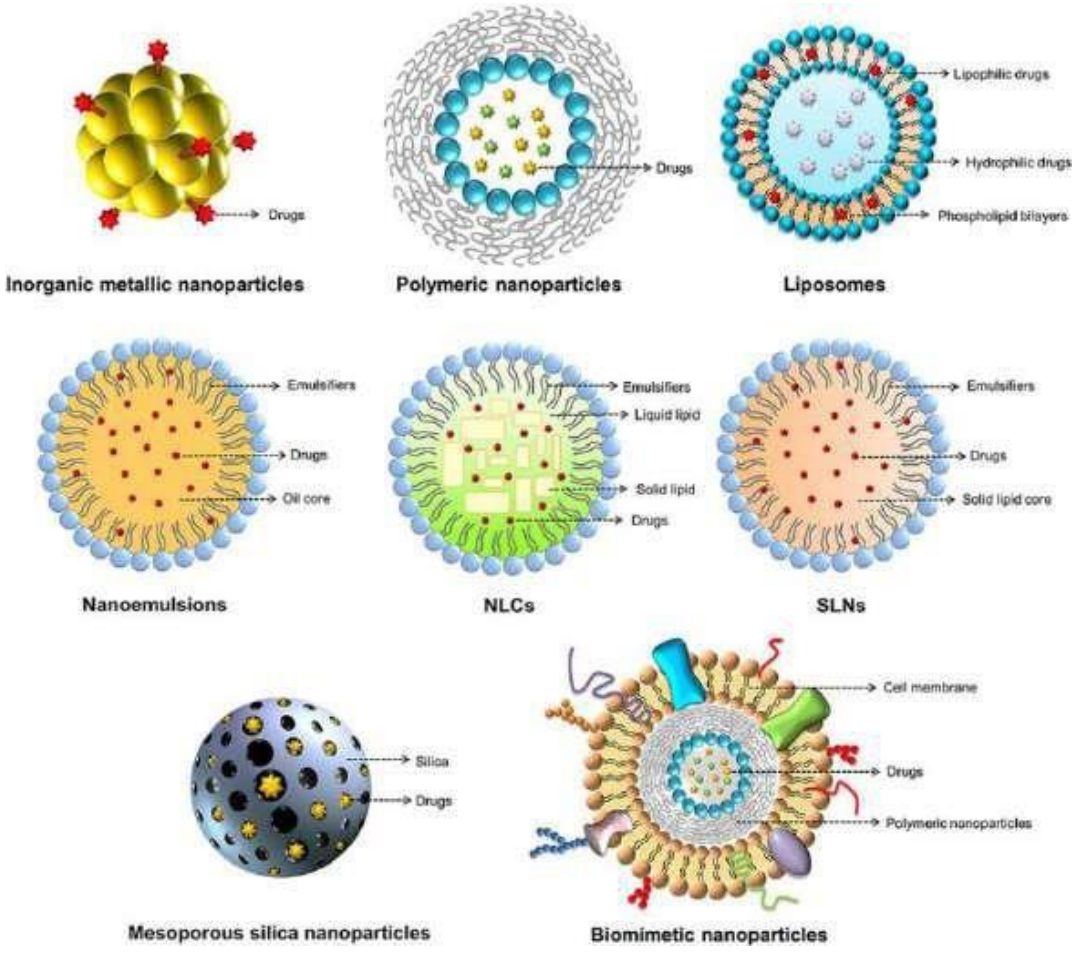


Figure 2: Several nanosized antibiotic delivery systems [45].

2.3.2 Lipid-based nanoparticles

Lipid-based nanoparticles (LBNs) have garnered significant interest in nano drug delivery systems among researchers in recent years. The preferred properties of these nanoparticles, such as their selective targeting, controlled release, biocompatibility, modifiable surfaces, and reduced toxicity, have been extensively explored for their antibiotic applicability [46]. Incorporating antibiotics into the LBNs has substantially advanced their antibacterial therapy as they protect against enzymatic destruction while increasing the drug interaction with the target bacteria [47, 48]. These nanoparticles disrupt the lipophilic bacterial cell wall and membrane and ultimately destroy the bacterial intracellular components [49]. The significant advantages of lipid nanoparticles (displayed in **Figure 3**) including SLNs [50], polymer-lipid hybrid nanoparticles (PLNs), and nanostructured lipid carriers (NLCs) [51] were compared to other lipids colloidal drug delivery systems such as liposomes [52] are their rigid morphology and high kinetic stability [53], as can be seen in **Table 1**.

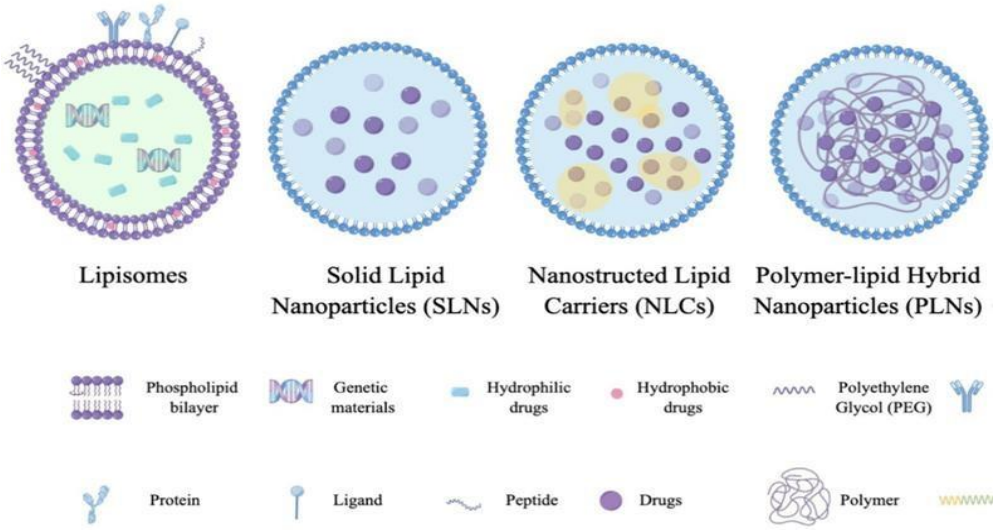


Figure 3: Structural differences of lipid-based nanoparticles [54]

However, achieving favourable LBNs can be a cumbersome process as their preparation includes numerous steps, including choosing appropriate lipids and their concentrations, methods of formulation, and surfactants if necessary [55]. The type of lipids required in these nanoformulations varies with the kind of nano delivery system and their desired therapeutic outcomes. Some LBNs are manufactured using diverse solid lipids with a low melting point at room and body temperatures, such as ascorbic acid, Compritol®, and Precirol® ATO5. While others are manufactured using liquid lipids such as oleic acid, castor oil and farnesol. Furthermore, an appropriate surface modification could result in enhanced targeting and thereby improve the overall efficiency of the drug. These NDDS are extensively studied due to their appealing characteristics such as cost-effectiveness, simplicity in preparation, and feasibility in large-scale production [56].

Table 1: Characteristics differentiating lipid-based nanoparticles.

Lipid carrier	Lipid composition	Structure and assembly	Properties	Ref
SLNs	Solid lipids	Nanosphere: solid lipids coated with a surfactant	Hydrophobic nature: burst drug release is common; low drug loading for hydrophilic antibiotics	[37, 57]
NLCs	Solid lipids and liquid lipids:	Nanosphere: a mixture of solid and liquid lipids with a surfactant coating	More sustained drug release; increased drug loading capacity	[51, 58]
Liposomes	Phospholipids	Nanocapsules: Phospholipid bilayers	Increased biocompatibility; high drug-loading; reduced toxicity	[55, 59]
PLNs	Phospholipids	Nanocapsule: Polymer core with lipid coat	Stable physical nature due to polymer matrix; potential in biofilm reduction	[60, 61]

2.3.3 Nanostructured lipid carriers

Nanostructured lipid carriers (NLCs) are a novel lipid-based nanoparticles designed to surmount SLNs deficiencies, such as the expulsion of drugs at long storage periods and poor drug loading capacity [58, 62]. NLCs are constituted by a solid and liquid lipids blend, leading to the less ordered lipid matrix, as depicted in **Figure 4** [63]. The imperfect crystal structure of NLCs provides the internal free space that allows the entrapped drug to remain inside the lipid matrix [64]. This type of nanocarrier can efficiently load both hydrophilic and lipophilic drugs. In hydrophilic drugs, the enhanced entrapment efficiency is due to the lipid conjugation, where the functional groups of the lipid and the drug are conjugated through carbodiimide [54]. Moreover, increased loading capacity in lipophilic drugs is due to the intrinsic property of most medicines to dissolve better in liquid lipids compared to solid lipids, as the enhancedsolubility maximizes the loading capacity [65].

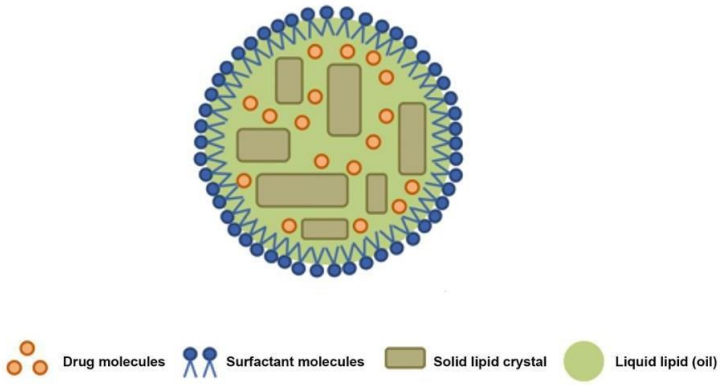


Figure 4: Primary structure of nanostructured lipid carriers [66]

NLCs comprise biocompatible/biodegradable solid and liquid lipids, and appropriate surfactants stabilize the nanoformulations. These lipids are used at specified concentration ratios between solid and liquid lipids ranging between 4:1 to 1:4, and surfactant concentrations range from 0.25 to 6% (w/v) [54]. The liquid lipids that are commonly used to prepare NLCs include oleic acid, miglyol 812, castor oil, and medium chain triglycrides. On the other hand, the solid lipids used include cetyl palminate, glyceryl tripalmitate, stearic acid, glyceryl palminostearate, and glycerol behanate, among others [67, 68]. NLCs are mostly stabilized by use of Tween 80/40/20, Poloxomer 188/407, and phosphatidylcholines. Additionally, physicochemical characteristics of NLCs are determined by their method of preparation. Furthermore, NLCs are classified based on their differing structures (presented in **Figure 5**), lipid composition, and concentration ratios into three types including:

Type I (Imperfect crystal)- is composed of different liquid lipids and solid lipids matrix, which has imperfections in the crystal order, providing free space for drug incorporation [69].

Type II (Amorphous)- is produced by the mixture of specific liquid lipids in a lipid matrix, forming an amorphous lipid matrix that prevents the extrusion of the drug, which improves the storage and delays the crystallization of lipids in the NLC formulations [70].

Type III (Multiple O/F/W) is formed by adding excessive liquid lipids to the lipid matrix. They will spread oil nanocompartments in the solid matrix, which acts as a barrier that prevents drug leakage and provides sustained drug release. Furthermore, the solubility of hydrophobic drugs is enhanced in oil nanocompartments [71].

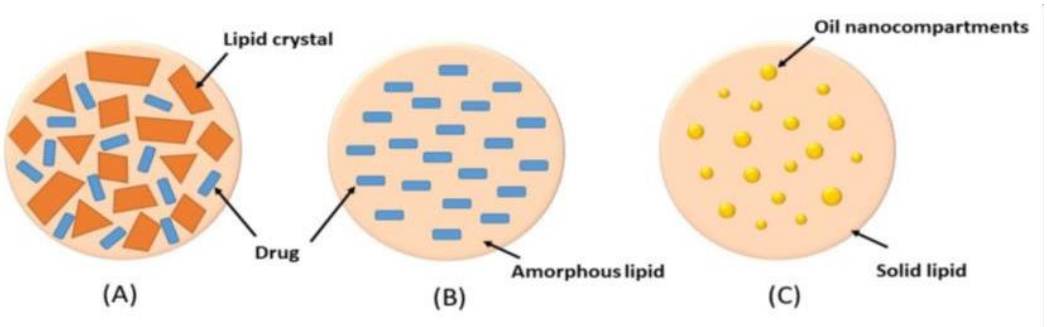


Figure 5: Types of NLCs based on structural differences [72]

2.3.3.1 Methods Applied for NLC Fabrication

Hot homogenization and ultra-sonication method

Hot homogenization and ultrasonication are the commonly applied techniques when preparing NLCs to achieve narrow particle distributions from lipid with high melting points [74, 75], and other favorable advantages such as their cost-effectiveness and reproducibility. In this technique, different phases of lipids are melted at a temperature 5-10 ℃ above the melting point of solid lipids, and the lipophilic drug is usually dispersed homogenously in the lipid phase. An aqueous phase consists of surfactant and hydrophilic drug which were incubated in the same temperature as the lipid phase is dispersed in the lipid phase. Thereafter, both phases are homogenously mixed using a high-shear mixer. Then, the droplets due to bubbles' growth, implosive collapse, and formation are broken down using ultrasonication [76]. The formation of NLCs is achieved by solidification at 2-3 ℃. This method successfully was applied to the fabrication of lycopene-loaded NLCs with particle size (PS) of 200 nm and polydispersity index (PDI) of 0.32, where molten monostearin and medium-chain triglycerides were homogenized followed by ultra-sonication [77]. Osman et al. (2019) [56] used a similar strategy to prepare vancomycin (VCM)- NLCs using stearic acid-derived solid lipids and oleic acid-derived liquid lipid with PS of 225.2 ± 9.1 nm and PDI of 0.258 ± 0.02 was obtained. Despite the favourable characteristicsof this method, its application is limited to thermolabile drugs and excipients.

High-pressure homogenization method

High-pressure homogenization (HPH) is a commonly applied method for NLC preparation as it demonstrates several advantages, such as the ability to produce small particle sizes, scale-up production, and short production time compared to the other methods [78, 79]. This can be applied both in cold and hot temperatures. For hot HPH, the lipid phase containing the drug if hydrophobic and lipids are preheated at temperatures 5 ℃ above the melting point of the solid lipid [80]. An aqueous phase consisting of surfactants and the hydrophilic drug is incubated in the same temperature as the lipid phase before dispersing both phases. After melting the lipid phase, both phases (at the same temperature) are homogenized using a high-shear mixing device, resulting in nanoemulsions, which are then cooled forming the NLCs through lipid crystallization [81]. For cold HPH, the drug is dispersed in molten lipids, and the mixture is cooled using liquid nitrogen or dry ice, providing a homogenous dispersion of drugs in lipid phase [82]. Subsequently, the drug-lipid mixture is milled and added in a surfactant solution at the same temperature[68, 83]. Hot HPH was successfully applied in the co-delivery of trimethoprim and sulfamethoxazole via NLCs. The aqueous phase at 75℃, which contained Tween 80, was added under stirring to the molten lipid phase at the same temperature containing lecithin and monostearin. The NLCs had PS of 198± 11 nm, PDI of 0.273± 0.011, and EE% of 86.2% [84]. In another study, cold high-pressure homogenization was applied to produce

ondansetron-NLCs using Tristearinmolten with Phosal® 53 MCT and stabilized using tween 80. The fabricated NLCs showed PS of 270 ± 6nm, 0.358 ± 0.028, and EE% 86.6 ± 3.0 [85]. The hot HPH is preferred over the cold HPH as it produces NLCs of smallersizes.

Microemulsion method

Microemulsion is one of the methods used to fabricate NLC, which has desired characteristics such as simplicity, reproducibility, scalability, avoiding organic solvents. In this method, the microemulsion is diluted in a cold aqueous solution, forming a nanoemulsion that develops NLC through lipid precipitation. In brief, a drug is dispersed in lipids that were heated above their melting points, and (at similar temperature) is poured under a vigorous stirring to develop a clear and thermodynamically stable microemulsion [86, 87]. The microemulsion is subsequently added into a cold aqueous phase under vigorous stirring [88]. NLCs are formed by lipid crystallization, which results from nanoemulsion formation upon dilution [82]. This method wassuccessfully applied in the preparation of methotrexate loaded NLC where a solid lipid mixture of stearic acid and Gelucire®50/13, and transcutol as liquid lipid showed small PS of 181.5 ± 11.5nm and low PDI of 0.118 [89]. The limitation of this method is the requirement for high surfactant concentrations, which are undesirable in formulations as they often lead to the recrystallization of NLCs[90].

Solvent Emulsification-Evaporation method

The solvent emulsification-evaporation method uses water-immiscible organic solvents such as dichloromethane, chloromethane, toluene, and cyclohexane in order to prepare NLCs [91, 92]. Briefly, an organic phase consists of the lipid and the drug dissolved in an organic solvent is emulsified in an aqueous phase, forming nanodispersions. The organic solvent is then evaporated using a rotatory vapor [93, 94]. In a study, Ranpise *et al*. (2014) [95] fabricated lercanidipine hydrochloride-loaded NLCs using this technique. Linseed oil, glyceryl monostearate, and lercanidipine hydrochloride were dissolved in an organic solvent at 80℃. An aqueous phase containing 1.5% Lutrol®F68 was used to emulsify the mixture at 80℃. Solvent was evaporated using mechanical agitation. The lercanidipine-NLCs [96] had a PS and EE%of 214.47 ± 0.3 nm and 71.57 ± 2.5%, respectively. In this study, the lipid concentration in the organic phase notably affected the NLC formation [90]. The solvent emulsification-evaporation technique is compatible with thermolabile drugs as it does not include heat. The limitation of producing NLCs using this method is that NLCs require purification as they include toxic organic solvents [97].

Solvent Emulsification-Diffusion method

The solvent emulsification-diffusion method uses water-miscible organic solvents such as butyl lactate, ethyl acetate, methyl acetate, isopropyl acetate, and benzyl alcohol are used to prepare NLCs. The initial thermodynamic balance between both phases is achieved by the mutually saturation of organic solvent and water. Prior the emulsification in the aqueous phase consisting of a diluted solvent and a surfactant, the lipids and drugs are dissolved in the diluted solvent under vigorous mixing to

form o/w emulsion. Subsequently, water is added to the emulsion in a volume ratio between 1:5 and 1:10 to enable solvent diffusion into the constant phase. Therefore, the NLCs are spontaneously formed due to the process of lipid crystallization, and the residual solvent expelled using either vacuum distillation or lyophilization [99, 100]. This method produced rivastigmine hydrogen tartrate-loaded NLCs from triacetin and Compritol 888 ATO dissolved in a mixture of ethanol and chloroform (1:1) dispersed with rivastigmine hydrogen tartrate forming a lipid solution. The lipid solution was added in the aqueous surfactant solution and ultra-sonicated. The resultant emulsion was dispersed in an aqueous medium containing Poloxomer 188 and was carefully stirred to evaporate the organic solvent. The produced NLCs has a PS 237 ± 1.36 nm and EE% of 53.34% [101]. However, this method requires purification to remove the residual organic solvent [102].

Phase Inversion Temperature (PIT) Method

The phase inversion temperature method is commonly applied in the fabrication of NLCs due to their advantages, such as improved stability, narrow size distribution, and low energy. In this method, the temperature activates inversions of water in oil (w/o) to oil in water (o/w) emulsions and inversely. Temperature sensitive non-ionic polyoxyethylated stabilizers are required in this method. Dehydration of the ethoxy group occurs when the temperature is raised, the hydrophobicity of the surfactant increases, and there would be a decrease of hydrophilic-lipophilic balance values of the surfactant [103]. PIT is known as the temperature which the affinities of the lipid and aqueous phase are at equilibrium [104, 105]. Primarily, the surfactants favour the development of w/o emulsions above PIT, whereas, below PIT, they reverse to the formation of o/w emulsions [106]. In developing NLCs, oils, water, and surfactants are incubated at a temperature above PIT under constant mechanical mixing for the formation of w/o emulsion. After that, these emulsions are immediately cooled with stirring, which then destructs w/o microemulsions leading to the formation of o/w nanoemulsions. Lipid precipitation at low temperatures results in the development of NLCs [107]. This method was employed in the preparation of NLCs loaded with metronidazole using glyceryl monocaprylate and monostearin. The resulting NLCs had a PS of 276.1 ± 4.36nm, PDI of 0.3, and EE% of 40% [108]. The disadvantages of applying this method to NLC production are the reduced stability of nanoemulsions formed and the requirement of several temperature cycles [109].

Solvent diffusion method

This method uses water-miscible solvents such as methanol, ethanol, isopropanol or acetone to dissolve lipids and drugs. In this method, the organic phase is rapidly dispersed using a needle into the aqueous phase, which contains an emulsifier and water or buffer solution, under stirringe [110]. After that, the solvent is allowed to diffuse from the droplets into the aqueous phase, thus reducing the size. Consequently, local supersaturated regions in the aqueous phase, which the surfactants stabilize, are formed due to increased lipid concentration within the droplets [111]. The surfactants functions to reduce the interfacial tension between solvent and water, leading to the formation of tiny droplets at the

injection site. Subsequently, these droplets are broken down into tinier ones that have the similar lipid concentrations due to interfacial turbulence and pulsation [111, 112]. The free energy released from the solvent is redistributed in order to reach the equilibrium phase is provided to the droplets [113]. The NLC formation is dependent on the emulsifiers, as the use of emulsifiers reduces the particle sizes and size distribution. The increase in emulsifier concentration reduces the PS and PDI until they reach the critical value (0.5-1.5%), where the PDI and PS increase due to a further rise of emulsifier concentrations. Notably, the diffusion rate of organic solvents is a key factor affecting size distributions and the PS [114]. This method was employed in the development of Ondansetron hydrochloride-loaded NLCs which had a PS of 185.2 ± 1.9 nm, PDI of 0.214 ± 0.006, and the EE % was 93.2 ± 0.5% [115]. The requirement of an additional procedure to remove the solvent residue limits the application of this method in fabricating NLCs.

2.3.3.2 Overview of NLCs for antibiotic therapy

Nanostructured lipid carriers have recently gained ground in the application of antibiotic therapy due to their desired advances in meeting the colossal demand for effective treatment strategies against bacterial infections. These antibiotics-loaded NLCs have demonstrated successful outcomes when administered via topical [116, 117], oral [118-120], parenteral [121, 122], and pulmonary routes [123, 124]. Jounaki *et al*. (2021) [125] conducted a study to enhance the antibacterial efficiency of VCM, formulated VCM-loaded NLCs from cholesterol and stearic acid as the bases of the lipid matrix and stearylamine using a cold homogenization technique. The study reported a particle size of 96.40 nm and a VCM encapsulation efficiency of 74.80%. The *in vivo* antibacterial study, using *S.aureus* and MRSA as test organisms, showed a 3-fold increase in the internalization of VCM-loaded NLCs to that of conventional VCM in the vitreous humor. Further, the biosafety evaluation presents the nanocarrier as the non-toxic carrier of VCM. These results concluded that the prepared NLCs could maintain the concentration of VCM higher than MIC, thereby improving the bioavailability for topical application and reducing side effects.

Another study by Shajari *et al.* (2020) [126] investigated the ability of NLCs to improve the antibacterial efficiency of curcumin against wild strains of *S.aureus*, *P.aeruginosa*, *E. coli*, and *Enterococcus faecalis.* NLC-curcumin was prepared using cetyl palmitate and black seed oil by hot homogenization method. This study reported the particle is 137.9 nm, and the encapsulation efficiency was 99.97%. The *in vitro* antibacterial evaluation of NLC-curcumin showed superiority over curcumin as it showed a minimum effective concentration of 0.125 μM, at which curcumin had no antibacterial effect due to the higher MIC of curcumin. Despite the favorable antibacterial efficacy of NLC-curcumin, biocompatibility evaluation of the formulated nanoparticles was necessary in this study to ensure the reduction of toxicity. Another study conducted by Youssef *et al*. (2020) [127] aimed to improve the ability of CIP to manage bacterial endophthalmitis by incorporation in NLCs. CIP-NLCs were

formulated from Precirol® ATO 5 and oleic acid by hot homogenization technique. The study reported a particle size and entrapment efficiency of 193.1 nm and 96.3%, respectively. These CIP-NLCs further loaded an *in situ* gel (CIP-NLC-IG) with a drug content of 94.8%. This study reported a sustained release profile of CIP-NLC-IG over 24 hours. This study concluded that CIP-NLCs could improve the bioavailability and prolong the antibacterial activity against bacterial infections.

Recently, the ever-stringent demand for novel nanocarriers to improve the delivery of drugs to the infection site has challenged researchers to exploit factors synonymous with the microenvironment conditions and mechanisms of resistance. As a result, Osman *et al*. (2019) [56] synthesized pH-responsive novel lipids (stearic acid-derived solid lipids and oleic acid-derived liquid lipids) which were used to prepare VCM-NLCs by hot homogenization/ultrasonication method to enhance antibacterial delivery. The study reported the VCM-SAOA-NLCs with a particle size of 225 nm and entrapment efficiency of 88.7%. Notably, VCM-NLCs showed increased antibacterial activity at pH 6.00. The group of researchers then concluded that VCM-loaded NLCs that respond to the pH condition of the bacteria could potentially enhance the antibacterial therapy against SA and MRSA infections.

In addition to the above, surface modification of nanocarriers has become a significant interest of researchers to improve the treatment of bacterial infections [128]. The surface of the nanocarriers can be potentiated using several materials, including polymers, peptides, antibodies, and oligonucleotides for application in diagnosis and targeted drug [129]. In a study reported by Suciati et al. (2018) [130], pH-responsive rifampicin-loaded NLCs were fabricated using solvent emulsification-evaporation technique using chitosan and acemannan for coating. The particle size, PDI, and entrapment efficiency were 301.4 nm, 0.28, and 90.9%, respectively. In this study, modification of NLCs with chitosan improved the antibacterial activity and drug solubility. In another study, Balguri et al. (2017) [131] studied the PEGylation of ciprofloxacin-loaded NLC. The group reported a particle size of 217 nm and entrapment efficiency of 84.2% on PEG (5K)-CIP-NLCs. Meanwhile, PEGylated NLCs showed 2-fold higher corneal permeation than free ciprofloxacin. The study above showed good permeation and controlled release of PEG-CIP-NLCs for enhanced corneal delivery. Despite the advantages of NLCs mentioned in the above reports, no study has explored their potential in targeting additional bacterial survival strategies.

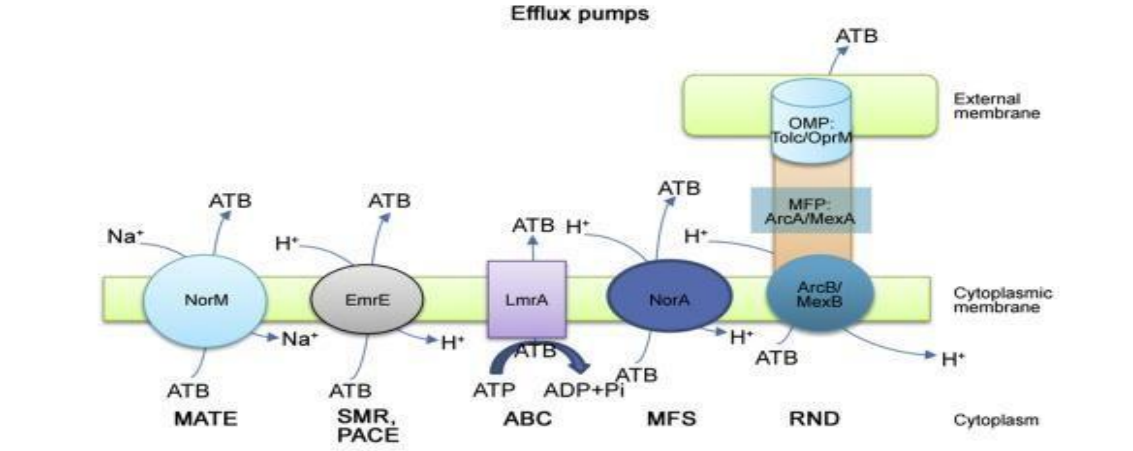
2.4 Efflux Pumps as a Mechanism of Antimicrobial Resistance

The challenges facing the successful treatment of bacterial infections are exacerbated by the increasingly growing antimicrobial resistance, rendering several classes of antibiotics ineffective. Bacteria can develop antimicrobial resistance through intrinsic mutation or horizontal gene transfer [132]. The mechanisms by which bacteria acquire antibiotic resistance comprise activation of the efflux pump, drug lysis by hydrolytic enzymes, drug modification [133], and drug suppression [134].

Activationof efflux pumps provides a first line of defense for bacterial survival against antimicrobial agents, posing a considerable concern to the globe as they are a primary cause of multidrug resistance [135, 136]. The overexpression of efflux pump systems is associated with the extrusion of wide-range antibiotics from the bacterial cells, thus reducing active drug concentration in the bacterial infection site, resulting in the subtherapeutic concentrations showing low to no effectiveness [137]. Among 30 reported efflux pump genes in *S. aureus* alone, NorA is the most studied gene, with a 43% possibility of overexpression, especially in MRSA [138, 139]. The NorA efflux pump has many substrates, including fluoroquinolones, quaternary ammonium compounds, biocides, antiseptics, and dyes [140].

2.4.1 Efflux pump mechanisms

These proteinaceous transporters are predominantly found in the cytoplasm of all bacterial cells, and they require energy to export unwanted substances from the bacterial interior to the exterior [141]. Efflux pumps are grouped into five major superfamilies based on their amino acid sequence and source of energy, as described in **Figure 6** [142]. These include:



MATE: Multidrug And Toxic compound Extrusion; SMR: Small Multidrug Resistance; PACE: Proteobacterial Antimicrobial Compound Efflux; ABC: ATP-Binding Cassette; MFS: Major Facilitator Superfamily; RND: Resistance Nodulation cell Division.
ATB: Antibiotic. MFP: Membrane Fusion Protein. OMP: Outer Membrane Protein.

Figure 6: Main mechanisms of efflux pumps [143]

2.4.1.1 Major facilitator superfamily

Major facilitator superfamily (MFS) transproteins are capable of extruding small molecules like drugs [144], anions [145], sugars [146], and metabolites of the Kreb cycle [147]. The structure of these proteins is made up of a single polypeptide chain consisting approximately 400 to 600 amino acids, and in certain instances, can include 12 to 14 transmembrane helices, one of which is amino, and the other is carboxyl-terminal [148]. This efflux pump class depends on proton motive force as an energy source. The transporters of this family are further grouped into 1) Sympoters can transport two or more molecules to one direction where driving force is used as the energy source; 2) uniporters transports a single molecule by the substrate gradient; and 3) antiporters transport two or more molecules in opposite directions on the plasma membrane [145, 149, 150]. The primary efflux pumps under this family such as *NorA*, *NorB*, and *QacA*, are mainly found in gram-positive bacteria. The role of these transporters is to transport andexpel antibiotics into the cell-extracellular medium [151].

2.4.1.2 Small multidrug resistance family and proteobacterial antimicrobial compound efflux

The small multidrug resistance and proteobacterial antimicrobial compound efflux families of efflux pumps demonstrate diverse transporters, structures, and functions, enabling them to release a wide range of drugs, dyes, and cations in the extracellular medium [152]. This diversity is attributed to the energysource these families use, which is proton driving force. This energy source induces the pumps within the plasma membrane, alerting when certain charged compounds enter the cells, providing protectionby expelling these undesired substances [153]. The proteins of these family are formed by homo-oligomeric complex which consists of 100 to 140 amino acid residues for the extrusion of molecules. The substrates of these proteins include several classes of antibiotics, quaternary ammonia compounds, and antiseptics [154, 155]. The main efflux pumps *QacA, QacB, QacG, QacH, Smr, EmrE, and MdtJI*in *Escherichia coli* [156, 157], and *SepA* in *Staphlococcus aureus* [158].

2.4.1.3 Resistance-nodulation-cell-division (RND) superfamily

The intrinsic resistance associated with these tripartite transproteins ascribe from the inner and outer membranes which is joined by the periplasmic space leading to the formation of outflow duct for noxious substances including antibiotics [159, 160]. RND proteins show structural differences from other subfamilies as they have more than 1000 identified amino acid residues and the protein inner membrane cells included two large periplasmic loops that split into two transmembrane segments, one of which included a protein fusion involving the C- and N-terminal domains [153, 161]. These proteins use proton gradient as an energy source to expel antibiotic classes such as fluoroquinolones, cephalosporins, tetracycline and chloramphenicol [162]. These efflux pumps are reported to be the primary mechanism for high drug resistance in Gram-negative bacteria. *MexAB-OprM* pump and *MexXY* are the common efflux pumps in *Pseudomonas aeruginosa* [163].

2.4.1.4 Multidrug and toxin extrusion superfamily

This family of efflux pumps uses electrochemical gradients of Na^+ and H^+ ions as energy source to export toxic substances such as fluoroquinolones, aminoglycosides, and dyes [164, 165]. This family of efflux pumps has a structural difference of 450,550 and 700 amino acid residues, consisting of 9 to 12 transmembrane helices in double domains, a single carboxyl-terminal and another amino [166]. The main efflux pumps under this family are *NorM, DinF,* and *MepA* [153]

2.4.1.5 ATP-binding cassette transporter superfamily

The ATP-binding cassette transporters are widespread membrane proteins with 52 subfamilies found in the plasma membrane and organelles that mediate the expulsion of drugs, amino acids, ions [167], and sugars uses hydrolysis of ATP as an energy source [152]. The structure of these proteins consists of approximately 1200 amino acids in four domains, two cytoplasmic hydrophilic nucleotides binding domains bound by two hydrophobic transmembrane domains organized in 6 α-helices [153, 168]. After the connection of the domains, structural changes result from the hydrolysis of ATP, and the

drug's translocation or efflux follows a signal from the two cytoplasmic hydrophilic nucleotides binding domains to the transmembrane domain until the cycle is reconstituted [151, 152]. As prominent members, this superfamily includes glycoprotein, *drrA*, *drrB*, and *drrC*.

2.4.2 Overcoming Efflux Mechanisms via Nanocarriers

Efflux pumps increase bacterial tolerance by expelling variable toxic substrates, including dyes, antibiotics, biocides, and monovalent cations. This resistance mechanism substantially reduces the concentration of structurally diverse antibiotics at the infection site. The bacterial exposure to subinhibitory concentrations of the drug results in the accumulation of mutation, which alters the structure of the binding site [167]. Primarily, multidrug-resistant bacteria are caused by the overexpression of efflux pumps and modifications in the antibiotic-binding sites, and the underlying diseases are associated with high fatalities. The ability of these transporter proteins to expel one or multiple classes of antibiotics out of the cellular milieu highlights the urgent need for the discovery of efflux pump inhibitors and eventually circumvent multidrug resistance [168]. As a result, numerous studies have been generated to find compounds capable of reversing this bacterial resistance mechanism [169]. Most of the studies evaluate the phenotypical efflux pump inhibition of different compounds using a cart-wheel assay where the efflux pump expressing bacterial strains are pre-treated with the compound at subinhibitory concentrations before exposing them to dye (e.g., ethidium bromide) for accumulation and resulting in fluorescence which would indicate efflux pump inhibition [170]. Cart-wheel assay depends on ethidium bromide being one of the common substrates for bacterial efflux pumps [171].

Efflux pump inhibitory nanocarriers have emerged as a promising strategy to overcome antibiotic therapy failures and antimicrobial resistance. Several reports have investigated the efflux pump inhibition of metal nanoparticles, including copper nanoparticles [172], silver nanoparticles [173], zinc oxide nanoparticles [174], and titanium dioxide nanoparticles [175], among others. In a study, Christena et al. (2015) [172] investigated the efflux inhibitory role of copper nanoparticles against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The findings of this study showed that copper nanoparticles at 0.25 x MIC inhibit efflux pumps and increase the intracellular concentration of ciprofloxacin. These results were consistent with the study conducted by Iqbal et al. (2019) [176], which showed the efflux pump inhibition of thiolated cobalt-doped Zinc oxide nanoparticles against *NorA* at a reduced concentration. These results were due to the binding of the nanoparticles to the active site of the efflux pump, thus blocking the excretion of substrates from bacterial cells. The hemolysis assay of this study showed dose-dependent non-toxicity to redblood cells. Although the latter study evaluated hemocompatibility, more biocompatibility tests are necessary when studying inorganic nanoparticles to ensure their safety on the vital organs.

Various plant-derived compounds are reported to have intrinsic inhibitory activities against efflux pumps from different bacterial strains [177, 178]. Interestingly, these compounds are reported to boast

excellent efflux pump inhibitory effects independent of their antibacterial activity [179]. Plant-derived compounds such as eugenol, trans-cinnamaldehyde, and reserpine are well-known to be capable of reversing efflux pumps [180, 181]. However, these compounds demonstrate efflux pump inhibition at high concentrations associated with toxicity concerns to the human cells. Therefore, recent interest to researchers is towards exploiting efflux pump inhibition capabilities of these compounds in nanocarriers to improve the intracellular concentrations of antibacterial agents, as summarized in **Table 2**. Salih et al. (2020) [182] investigated the efflux pump inhibitory potential of tocopherol succinate for enhanced vancomycin delivery using nanocarriers. The study reported that self-assembled drug delivery (SADD) formulated using tocopherol succinate as an excipient showed inhibition of NorA and NorB, improving the delivery of vancomycin. The findings of this study showed the potential stable interaction of tocopherol succinatewith NorA and NorB at a molecular level using computerized tools, which indicated potential in the efficient delivery of vancomycin and enhanced antibacterial activity [183]. A subsequent study generated by Ibrahim et al. (2021) [184] evaluated the efflux pump inhibitory activity of tocopherol succinate in SLNs formulated with both tocopherol succinate and ascorbic acid as excipients for the improved delivery of vancomycin to the bacterial infection sites. The finding of this study showed superior interaction between NorA and NorB when compared to the previous report on SADD, potentially due to the presence of ascorbic acid in the nanocarrier. These studies have demonstrated the increased potential of lipid-based nanoparticles in inhibiting efflux pumps to enhance the intracellular concentration of antibiotics. The above studies performed biocompatibility tests that showed non- hemolytic activities and biosafety. To date, few studies have explored these compounds' efflux pump inhibitory capabilities in fabricating nanocarriers that include SLNs, self-assembled drug delivery, chitosan nanoparticles, and nanocapsules. Therefore, identifying other compounds with efflux pump inhibitory capabilities for more nanocarrier optimization is still required.

Table 2: Overview of efflux pump inhibitory nanosystems

Nanoparticle	Target efflux pump	Main findings	References
Chitosan	MexEF and OprN	The MIC of the efflux pump expressing strain was higher than that of strains that are reported to have no efflux pumps.	[185]
SLNs	NorA and NorB	*In silico* studies showed the increased potential of binding between ascorbyl tocopherol succinate and NorA and NorB.	[184]
Self-assembled drug delivery	NorA and NorB	*In silico* studies showed that tocopherol succinate has the potential to bind in the hydrophobic binding pocket and inhibit NorA and NorB.	[182]
Nanocapsules	NorA, NorB, MexAB, and OprM	The inhibition of efflux pumps in bacterial cells that were treated with nanocapsules was indicated by the fluorescence due to EtBr accumulation.	[186]

2.5 Ciprofloxacin as a model drug for antibiotic therapy

Ciprofloxacin (CIP) (structure displayed in **Figure 7**) is a widespread second-generation member of the Fluoroquinolone class of antibiotics used to effectively treat life-threatening bacterial infections like MRSA since 1987 [187]. CIP is frequently used in respiratory infection [188], urinary tract infection [189], external ocular infections [190], and otitis media treatment [191]. This broad-spectrum bactericidal antibiotic prevents DNA replication when administered against multiple aerobic gram-negative and gram-positive bacteria. However, CIP is progressively showing treatment failures towards different bacterial infections due to their resistance profile [192]. Therefore, there is a need to develop strategies to enhance the activity of this drug.

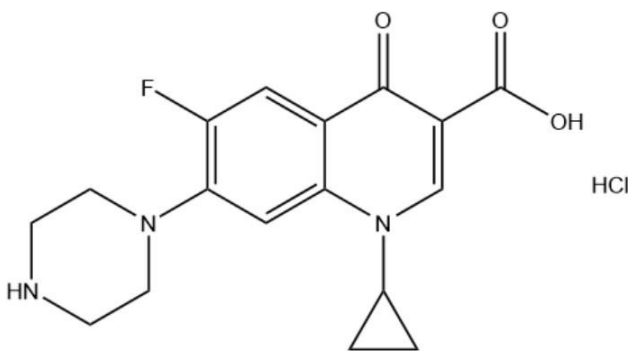


Figure 7: Chemical structure of ciprofloxacin (one-cyclopropyl-6-fluoro-4-oxo-7-(piperazine-1-yl)-1,4-dihydroquinoline-3-carboxylic acid) (drawn using ChemDraw)

2.5.1 CIP Mechanism of Action and Current Limitations

CIP acts as other molecules under the class of quinolones; they noncovalently bind to DNA gyrase (topoisomerase II and topoisomerase IV), as presented in Figure 8 [193]. DNA gyrase can catalyze ATP-dependent DNA supercoiling by a double-strand DNA break mechanism [194]. DNA gyrase consists of subunits A and B, and CIP and other quinolones are reported to selectively target DNA gyrase subunit A (gyrA) and exert its bacteriostatic activity. This drug prevents DNA re-ligation and ends with single-stranded DNA complexes, which may lead to exonucleolytic degradation and further terminate DNA replication [195].

On the other hand, the widespread resistance against several antibiotics is highlighted as a significant public health concern, causing difficulties and limitations in treating infectious diseases. The selective targeting of DNA gyrase and topoisomerase IV is attributed to the conservation of protein sequence between the two enzymes in the quinolone resistance determining region (QRDR) [196]. However, the spontaneous point mutations in the QRDR progressively impair the binding sites of CIP [197]. In addition, most Gram-positive bacterial strains exhibiting ciprofloxacin resistance express the excessive NorA gene [198]. Therefore, the primary resistance mechanism against ciprofloxacin is mutation and the overexpression of efflux pumps. This challenge highlights the need for innovative strategies to counteract these mechanisms and improve CIP efficacy.

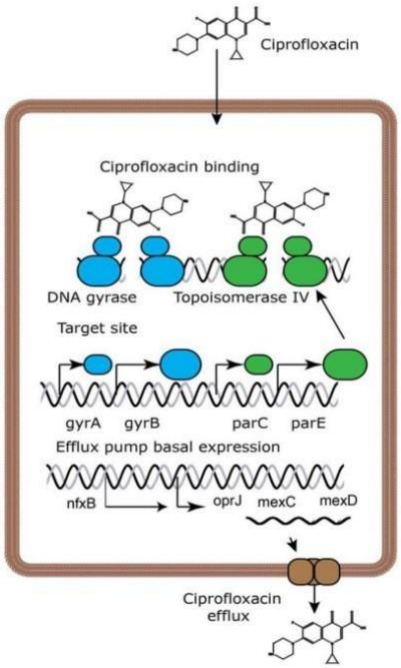


Figure 8: Ciprofloxacin mechanism of action and expulsion by overexpression of efflux pumps [189]

2.5.2 Overview of CIP nanosystems

The limitations mentioned above, coupled with poor solubility and low bioavailability, reduce the drug concentration of CIP at the infection site, necessitating the administration of large doses [194]. However, frequent doses of this drug can result in fatal double-strand DNA to affected cells [193]. To this end, CIP-loaded nanoparticles have been extensively studied for their potential to enhance antibacterial activity and biofilm reduction of CIP against different bacteria [185]. Several studies have investigated the antibacterial activity of CIP in various inorganic nanoparticles such as calcium carbonate [186], zeolite [187], silver [188], iron and zinc [189], selenium [190], silicon nanoparticles [191], among others. However, organic nanocarriers are the preferred choice to load ciprofloxacin due to their characteristics, such as increased loading efficiency, enhanced antibacterial activity, and reduced toxicity [33]. Although extensive research has been conducted on improving the delivery of CIPusing nanocarriers (as shown in **Table 3**), more strategies to enhance the efficacy of this drug are still required.

Table 3. Examples of CIP-loaded organic nano delivery systems

Nanocarrier	**Entrapment efficiency (EE%)**	**Main findings**	**Reference**
Cubosomes	75%	The antibacterial activity of this nanosystem was found to be 2.54-fold superior to *S. aureus.*	[193]
Niosomes	79.25%	CIP-loaded niosomes showed 4 to 5-fold MRSA reduction. This nanosystem shows potency to eliminate biofilms.	[194]
Nanoemulsions	-	CIP-nanoemulsion showed similar antibacterial but improved antibiofilm against *P.aeruginosa*, *S.aureus*, and *E.coli*.	[195]
SLNs	73.94%	CIP-SLNs showed superior antibacterial activity against *S. aureus* and *P. aeruginosa* over CIP.	[197]
Capsules	86.90%	CIP-capsules showed improved antibiofilm activity. However, the antibacterial activity was comparable to that of unloaded CIP.	[198]
Hydrogels	93.8%	These nanocarriers showed excellent antibacterial activity but were not compared to free drugs.	[199]
Micelles	-	Micelles loaded with CIP showed a 4-fold higher antibacterial effect than free CIP.	[200]
PLNs	82.7%	CIP-PLNs had increased activity against *S. aureus* and *P.aeruginosa.* This system also presented biosafety to infected tissues/cells.	[201]
NLCs	84.2%	CIP-NLCs had 2.74-fold increased antibacterial activity than CIP (free)	[136]

2.6 Conclusion

The chapter highlighted the literature findings on the current state of bacterial infections and therapy failures due to the continuously emerging mechanisms of antibacterial resistance. It also outlines the potential of efflux pump inhibitory nanocarriers to increase the intracellular accumulation of antibiotics and combat multidrug resistance. Despite the extensive research in lipid nanoparticles, only few studies have explored their potential as efflux pump inhibitors. Furthermore, the chapter has presented ciprofloxacin as a model drug associated with therapeutic failures and highlighted the need for innovative strategies to enhance their efficacy.

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Chapter Three

Manuscript

3.1 Introduction

This chapter addresses the aims and objectives stated in **Chapter 1**, and it is a first-authored experimental article to be submitted to the journal for publication. This article presents optimized ciprofloxacin-loaded nanostructured lipid carriers (CIP-NLCs) with multi-functional therapy against bacterial infections. *In vitro* studies efflux pump inhibitory capabilities of CIP-NLCs against efflux pumps associated with *P. aeruginosa* and MRSA investigated. *In* addition, different characterizations performed on the formulation, such as *in vitro* drug release, *in vitro* biosafety, and *in vitro* antibacterial and antioxidant properties are discussed.

3.2 Author contributions

In this paper, Mr. Sbongumusa Dlamini contributed to the project design and characterisation of the nanocarrier. In addition, Mr Dlamini was responsible for the analysis and interpretation of all data, wrote the first draft of the paper and addressed all the corrections from the co-authors. Mrs. Eman Elhassan assisted with *in vitro* antibacterial activity studies, analysis, and interpretation of the data. Miss Eman A. Ismail assisted with bacterial kinetics test and *in vitro* antioxidant activity. Mr Mohammed A. Gafar assisted with Transmission Electron Microscopy. Dr Xylia Peters contributed to the editing of this document. Ass. Prof Calvin A. Omolo assisted with the conceptualization, design of the project, problem-solving, thesis editing and supervision of all the studies. Prof Thirumala Govender served as supervisor and was responsible for project conceptualization, thesis and abstract editing and overall supervision of the study.

3.3 Manuscript

Formulation of nanostructured lipid carriers using eugenol and D-α-tocopherol succinate for the inhibition of bacterial efflux pumps to enhance delivery of ciprofloxacin.

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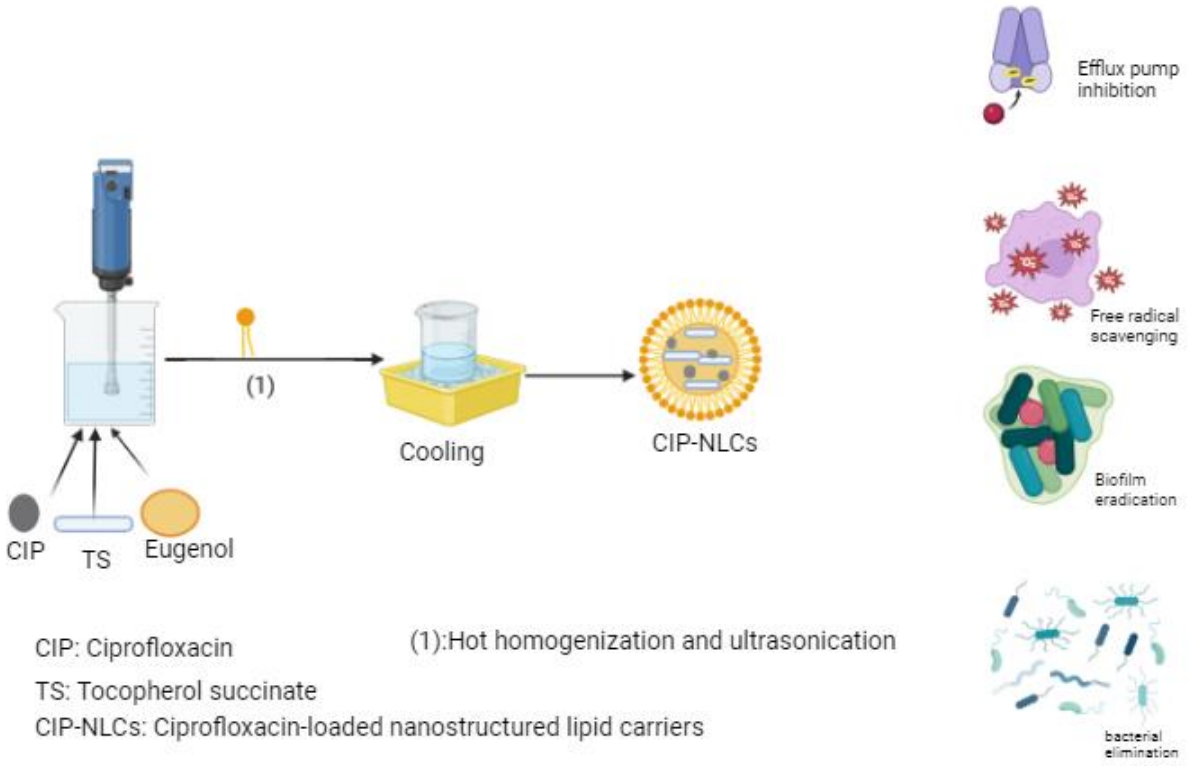
Abstract

In the present study, hot homogenization and ultrasonication method was employed in the formulation of ciprofloxacin-loaded nanostructured lipid carriers (CIP-NLCs) with efflux pump inhibitory activities using D-α-tocopherol succinate (TS) and eugenol to improve the antibacterial efficacy of ciprofloxacin against multidrug-resistant bacteria. CIP-NLCs had a particle size, polydispersity index, surface charge, and entrapment efficiency of 147.4 ± 0.59 nm,
0.219 ± 0.009, -9.64 ± 2.22 mV, and 82.8 ± 0.39 %, respectively. The *in vitro* biosafety evaluation revealed CIP-NLCs as non-haemolytic. The *in vitro* drug release study showed a biphasic release of CIP from the CIP-NLCs for 48 hours at pH 7.4. The *in vitro* antibacterial activity of CIP-NLCs showed 2-fold lower minimum inhibitory concentration values compared to bare ciprofloxacin against Methicillin-resistant *Staphylococcus aureus* (MRSA)(CIP-NLCs:12.5 μg/mL and CIP: 25 μg/mL), *Staphylococcus aureus* (SA)(CIP-NLCs:0.195 μg/mL and CIP: 0.39 μg/mL), *Escherichia coli* (*E. coli*)(CIP-NLCs:0.048 μg/mL and CIP:0.195 μg/mL), and *Pseudomonas aeruginosa (P. aeruginosa*)(CIP-NLCs:0.097 μg/mL and CIP:0.39 μg/mL). The bacterial-killing kinetic test showed 100% elimination of MRSA and *P. aeruginosa* within eight and one hour(s) of treatment with CIP-NLCs, respectively. Conversely, 100% elimination of MRSA and *P. aeruginosa* was shown within 24 and 12 hours of treatment with bare ciprofloxacin, respectively. CIP-NLCs eliminated 3-fold MRSA biofilm compared to bare ciprofloxacin, whereas 1.25-fold *Pseudomonas aeruginosa* biofilms were eliminated. Efflux pump inhibition potential of CIP-NLCs was confirmed using cartwheel assay, which showed high fluorescence intensity on bacteria treated with CIP-NLCs. The DPPH scavenging assay of CIP-NLCs proved antioxidant activity equivalent to Vitamin C (ascorbic acid), which is reported to be a potent antioxidant. The *in vivo* systemic infection in BALB/c mice reduced MRSA infection in kidney, liver, and blood by 12.27-fold, 4.47-fold, and 1613-fold, respectively. Therefore, the CIP-NLCs may serve as a promising tool for enhancing antimicrobial activity and overcoming resistance mechanisms againstMRSA and *P. aeruginosa*.

Keywords: Ciprofloxacin-loaded nanostructured lipid carriers D-α-tocopherol succinate; eugenol; efflux pumps;bacterial infections.

Graphical abstract



1. Introduction

Bacterial infections continue to be a severe global public health concern due to their high morbidity and mortality rates, challenging human resourcefulness [1]. Due to the growing crisis of antibacterial resistance, most previously available antibacterial therapies that effectively treat bacterial infections are becoming obsolete. Worldwide, antimicrobial resistance is reported to contribute to approximately 700,000 deaths annually [2, 3]. In addition, the emergence of bacteria resistant to broad antibiotics because of their uncontrolled and rampant use has further decreased the number of clinically available antibiotics [4]. Consequently, infections of multidrug-resistant bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (*P. aeruginosa)* are associated with exorbitant treatment costs and increased mortality, adversely affecting the quality of life [5]. Although ciprofloxacin is the most effective and widely used antibiotic in treating *P. aeruginosa* infections [6, 7], MRSA infections are reportedly resistant to this antibiotic [4]. According to the recent systematic review in the Lancet, MRSA infections alone constituted 100,000 worldwide deaths in 2019 [8, 9]. Thus, novel strategies to restore antibiotic efficacy for improved therapeutic outcomes towards these infections are urgently needed.

Since the initial discovery of antibiotics, conventional dosage forms such as tablets, capsules, and suspensions have been the only mode of delivery. However, despite the initial success in bacterial eradication, these therapies are associated with several disadvantages leading to suboptimal

concentrations at the infection sites, including reduced solubility and absorption, lack of targeting, short-half life, and low therapeutic index [10]. These limitations, uncontrolled dosing, and misuse of antibacterial agents are the primary components leadingto the challenge of antibacterial resistance [11]. Further, this challenge contributes to the poor management of bacterial infections for successful therapeutic procedures such as chemotherapy, transplant, and dialysis [12].

In addition to the above challenge, efflux pumps and biofilm formation provide bacteria with an additional survival strategy, increasing resistance to the current antibiotics [13]. Efflux pumps are membrane transport proteins in bacterial cells that prevent intracellular accumulation of a broad range of toxic substances, such as antibiotics and dyes, thus leading to multidrug resistance [14]. Moreover, efflux pumps are further implicated in bacterial behavioural factors, including biofilm formation [15], virulence, pathogenicity [16], and quorum sensing [17]. Due to the broad targets of efflux pumps, inhibition of bacterial efflux pumps could enhance the therapy armamentarium against resistant bacteria [18]. To this end, extensive research has been conducted over the past five years to combat efflux pump-mediated resistance using synthetic, natural-derived, and phage-mediated efflux pump inhibitors to restore antibacterial efficacy [19]. These efflux pump inhibitors are required at higher concentrations to achieve their maximum functions. However, the high concentrations of these compounds lack clinical applicability as they might have toxic side effects on the unaffected cells. Moreover, biofilms are matrix-encased bacterial communities associated with tolerance to lethal antibiotic concentrations. Eradication of biofilms necessitates high and multiple doses of the last-resort antibiotics [20]. Therefore, optimal delivery strategies for inhibiting these resistance mechanisms are urgently needed.

Novel nanosized drug delivery systems (NDDS) have showcased superiority compared to current dosage forms with an increased potential to enhance antibacterial therapies and combat antibiotic resistance [21]. Nano antibiotic delivery systems are associated with improved pharmacokinetics and pharmacodynamics properties and reduced risk of toxicity. Regrettably, despite the advantages of antibiotic nanocarriers, efflux pumps still expel a wide range of antibiotics, decreasing their active intracellular concentrations at the bacterial infection sites. Therefore, the application of NDDS as an anti-virulence strategy used to target biofilm formation and efflux pumps has been conspicuous to researchers in an attempt to combat antibacterial therapy failures and associated antibiotic resistance [22].

Efflux pumps are presented in variable classes based on their amino acid sequence and source of energy. Among other efflux pump families, the major facilitator superfamily (MFS) and resistance-nodulation-division (RND) family cause multidrug resistance in MRSA and *P. aeruginosa*, respectively. *NorA*, *NorB*, *AcrA*, and *MexA* are the common efflux pumps with many substrates, such as fluoroquinolones, biocides, quaternary ammonium compounds, antiseptics, and dyes [14]. Therefore, inhibition of efflux pumps with an increased potential of causing multidrug resistance has been a subject of significant

interest to different research fields with efforts to achieve a high active antibiotic concentration at bacterial infection site [23]. Several studies have reported the efflux pump inhibition capabilities of different nanocarriers, including silver nanoparticles [24], titanium dioxide nanoparticles [25], and copper nanoparticles [26]. However, these reports lack biocompatibility studies, which are crucial for highlighting the safety of these nanocarriers to healthy body cells/tissues. Therefore, optimized efflux pump inhibitory nanocarriers with low toxicity are still required.

Medicinal plants are reported as a rich source of compounds that boast efflux pump reversal capabilities and reduced toxicity, such as reserpine [27], eugenol, and trans-cinnamaldehyde [28], among others [29]. However, efflux pump inhibitory activities of these compounds are induced at high concentrations, increasing the risk of dose-dependent toxicity. Therefore, efflux pump inhibition of these compounds has been successfully employed in the formulation of nanocarriers such as solid lipid nanoparticles (SLNs) [31], nanocapsules [32], self-assembled drug delivery [33], and chitosan nanoparticles [34]. Moreover, nanostructured lipid carriers (NLCs) have attracted significant attention in nanotechnology due to their advantages, including high encapsulation efficiency and stability during long periods of storage. NLCs, which are second-generation lipid-based nanoparticles, consist of a blend of solid and liquid lipids, resulting in an imperfect crystal structure [35]. Due to the nature of NLCs, different compounds with capabilities to target different efflux pumps can be used in their formulation. Despite these desired characteristics of NLCs in delivering antibiotics, no study has explored their potential for inhibiting efflux pumps. Therefore, it is necessary to effectively identify compounds with capabilities of inhibiting efflux pumps using NLCs.

Herein, we propose the formulation of NLCs using eugenol as a liquid lipid and D-α-tocopherol succinate (TS) as a solid lipid. Eugenol is a phenylpropanoid derived from clove oil that improves cell membrane permeability and destroys the bacteria's plasmatic membrane [36]. Eugenol can enhance the efficacy of conventional antibiotics through their synergistic activity with drugs [37]. Most biological activities associated with eugenol are due to their antioxidant abilities owing to the phenol group in their structure [38]. Eugenol is reported to possess efflux pump inhibitory activities against *MexA* and *AcrA* which then suggests an enhanced the antibiotic sensitivity associated with this compound against *P. aeruginosa* and *E. coli* [39]. To date, no study has explored the anti-efflux capabilities of eugenol in a nanocarrier. Conversely, TS is a well-reported vitamin analogue composed of signalling, functional, and hydrophobic domains [41] which is documented to have antioxidant activities [40]. This lipid-soluble isoform enhances cellular penetration and uptake of substances such as antibiotics due to their hydrophobic domain, which facilitates the docking of tocopherol molecules to the bacterial membranes [40]. Modification of bacterial membrane permeability leads to their damaged intrinsic elements and the collapse of protons. Furthermore, efflux pump inhibition of TS has been investigated, specifically in two nanocarriers against *NorA* and *NorB*, to improve the intracellular accumulation of vancomycin [30, 33]. In addition to the efflux pump inhibition of TS, these studies also reported antibacterial and antibiofilm properties, and biocompatibility of these nanocarriers. TS and eugenol are

also reported to possess antioxidant activities in a microenvironment [40]. Thus, combining TS and eugenol would provide the NLCs formulation with efflux pump inhibitory and antibacterial effects.

Therefore, this study reports the formulation of CIP-NLCs from eugenol and TS to explore their efflux pump inhibitory and antibiofilm activities against MRSA and *P. aeruginosa* compared to bare CIP. Furthermore, the *in vivo* anti-MRSA activity was conducted using the systemic mice infection model. To our knowledge, this is the first report to explore eugenol in a nanocarrier for its efflux pump inhibition. Furthermore, no other study has reported the formulation and characterization of efflux pump inhibitory NLCs.

2.0 Materials and Methodology

2.1 Materials.

D-α-tocopherol succinate, eugenol, Tween 20, Span 80, Poloxamer 188 dialysis tubing cellulose membrane, Muller Hinton Broth 2 (MHB), crystal violet, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (USA). Muller Hinton Agar (MHA) and Nutrient Broth were purchased from Biolab Inc. (South Africa). Milli-Q purified water was obtained from an Elix® water purification system, Millipore Corp. (USA). Sheep blood was purchased from United Scientific SA cc. (South Africa). SA (ATCC 25923), MRSA (ATCC 700699), *P. aeruginosa* (ATCC 35218), *E. coli* (27853), and CIP hydrochloride were obtained from DLD Scientific (South Africa). The reagents and solvents used in this study were of analytical grade.

2.2 Preparation and screening of NLCs

Nanostructured lipid carriers were prepared using a previously reported hot homogenization and ultrasonication method [42]. The lipid phase containing eugenol and TS in varying ratios (1:1, 1:2, or 2:1) (m/v) was mixed and melted at 80 °C. Separately, the aqueous phase was prepared by dissolving varying lipid: surfactant ratios (1:1:0.5, 1:1:1, or 1:1:1.5) (m/v) of either Tween 20, Span 80, or Poloxamer 188 in 20 mL distilled water before heating at the same temperature. Thereafter, both phases were mixed and homogenized at 6000 rpm with an Ultra-turrax T-25 homogenizer (IKA Labortechnik, Germany) for 15 minutes. The resulting emulsion was subjected to sonication (30 % amplitude) for 15 minutes beforecooling to 20 °C. Several variables, such as lipid ratio, surfactant type (Tween 20, Span 80, and Poloxamer 118), and concentrations, were screened to obtain an optimum formula with reduced particle size, narrow polydispersity index (PDI), and acceptable zeta potential (ZP). After receiving the optimum formulation, CIP-loaded NLC formulations were also prepared using the same technique by adding 10mg of CIP in the aqueous phase, and entrapment efficiency was achieved. Subsequently, the produced NLC formulation (3 mL) was loaded into Amicon® Ultra-4 filter centrifuge tubes (Millipore Corp., 11USA), with a pore size of 10 kDa followed by 4500 rpm (25 °C) centrifugation for 20 minutes. The samples were reconstituted three times in distilled water to separate the CIP-NLCs from the free drug and unbound materials. All formulations were prepared in

triplicate.

2.3 Characterisation of NLCs

2.3.1 Determination of particle size, PDI, ZP, and morphology

The average particle size, PDI, and ZP measurements of the prepared NLCs were carried out by dynamic light scattering (DLS) using Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK). This was done in triplicate at a temperature of 25°C. Before measurement, the CIP-NLCs were diluted to tenth dilution factor with phosphate-buffered saline (PBS) (pH 7.4). The surface morphology of CIP-NLCs was studied via a Transmission Electron Microscope (TEM, JEOL 2100). Samples were placed on a TEM grid, stained with 1% uranyl acetate, and viewed after air-drying. TEM was operated at an accelerating 100 kV voltage[43].

2.3.2 Quantification of CIP in CIP-NLCs

Determination of the amount of CIP in CIP-NLCs was achieved by measuring entrapment efficiency (EE%), which was carried out using an ultrafiltration method. The CIP-NLCs, separated in section 2.2, were broken down using methanol and sonicated for 10 minutes. The unentrapped CIP in thecollected filtrate and CIP from the broken-down system were quantified using the High-Pressure Liquid Chromatography (HPLC) method using a Shimadzu (Japan) model and UV detector wavelength of 276 nm. The analysis was carried out using a mobile phase of acetic acid (2.5% v/v) in water and acetonitrile: methanol (1:1) at a flow rate of 1.5 mL/min, which was pumped through Nucleosil 100-5 C18 (250 mmx 4.6 mm) [44]. The EE % of CIP-NLCs was quantified using the following formula:

$$EE\% = \left(\frac{weight\ of\ CIP\ in\ nanoparticles}{Weight\ of\ CIP\ added}\right) \times 100 \quad (1)$$

2.4 Biosafety studies

2.4.1 Hemocompatibility

The haemolytic activity of CIP-NLCs was evaluated using a previously reported method [45]. In order to extract red blood cells, fresh sheep blood was washed three times using PBS (pH 7.4) and centrifugation for five minutes. The concentration of CIP-NLCs ranging between 0.05 to 0.5 mg/mL was obtained via serial dilutions usingPBS. After that, 1.8 mL of each sample was mixed with 0.2 mL of the red blood cells and incubated for30 min at 37°C. Afterwards, the samples were centrifuged at 10,000 rpm for 10 minutes to remove anyremaining red blood cells. Then 200 µL of the supernatant was carefully collected and seeded into 96- well plates and analysed for haemoglobin released by lysed cells at 540 nm using a microplate spectrophotometer (Spectrostar Nano, Germany). The positive and negative controls were prepared byadding 0.2 mL of the red blood cell suspension to 1.8 mL PBS (7.4) and distilled water, respectively. The percentage of haemolysis was calculated using the following equation:

$$\%\text{Haemolysis} = \left(\frac{ABS - ABS0}{ABS100 - ABS0}\right) \times 100 \quad (2)$$

Where ABS_{100} and ABS_0 are the absorbances of the solution at 100% and 0% haemolysis, respectively.

2.5 *In vitro* drug release study

The *in vitro* drug release studies of the encapsulated CIP from the fabricated NLC were performed using the dialysis release method at pH 7.4, as previously reported [46]. In brief, a dialysis bag (pore size cut off: 14 000 Da) containing 2 mL of the bare CIP, blank-NLCs, and CIP-NLCs, was placed in 40 mL of the release medium (PBS) and kept in a 100 rpm shaking incubator at 37 °C. At predetermined time intervals of 0.5, 1, 2, 3, 4, 5, 6, 8, 10,12, 24, and 48 hours, 2 mL of the sample was collected from the receiver compartment, and an equivalent amount of PBS was replaced to maintain the sink conditions. The amount of CIP released was quantified using the HPLC method using a similar protocol as mentioned in 2.3.2 [47].

2.6 Release Kinetics modelling study

The drug release data was then numerically analysed using the DD Solver program (DD Solver 1.0), an Excel add-in (Excel 2021), byfitting to a variety of kinetics models, including Higuchi, zero-order, Korsmeyer-Peppas, Hixson- Crowell, Weibull, and first-order models. The release mechanism was then described using either Korsmeyer-Peppas or Weibull exponent [48].

2.7 Antibacterial efficacy studies

2.7.1 *In vitro* antibacterial activity

The antibacterial activity of blank-NLCs, bare CIP, and CIP-NLC was evaluated by the micro broth dilution method against SA, MRSA, *E. coli*, and *P. aeruginosa* [49]. All bacterial cultures were grown in nutrient broth and kept in a 100 rpm shaking incubator at 37 °C. After 16 hours, the bacterial concentration was equilibrated to 0.5 McFarland's standard (DEN-1B densitometer, Latvia). Bacterial suspensions were diluted to 5 x 10^5 colony-forming units (CFU)/mL using Muller-Hinton broth (MHB) (7.4). CIP-NLC, bare CIP, and blank-NLCs were serially diluted from 50 to 0.0975 µg/mL in MHB. Bacterial suspensions were added to the plates and placed in a 37 °C shaking incubator at 100 rpm for 24 hours. After 24, 48, and 72 hours of incubation, the samples were spotted in triplicates on Muller-Hinton agar (MHA) plates and incubated at 37°C for 24 hours to determine minimum inhibitory concentration (MIC) values.

To gain more insight into the overall antibacterial activity of the blank-NLCs and CIP in the CIP-NLCs combinations, the Fractional Inhibitory Concentration (FIC) index was determined. The ΣFIC was calculated and analysed based on the results (MIC values) obtained from the *in vitro* antibacterial study using the following equations.

$$\text{FIC (CIP)} = \frac{\text{(the MIC of CIP in combination with blank}-\text{NLCs)}}{\text{(the MIC of CIP)}} \quad (3)$$

$$\text{FIC (blank-NLCs)} = \frac{\text{(the MIC of blank−NLCs in combination with bare CIP}}{\text{(the MIC of blank−NLCs)}} \quad (4)$$

2.7.2 Bacterial killing kinetics

Bacterial killing kinetics of CIP-NLCs and bare CIP against MRSA and *P.aeruginosa* were performed using the colony count technique for analysis [50]. MRSA cultures were prepared by diluting an overnight culture in nutrient broth and incubating for 16 hours. Thereafter, 5 x 10^5 CFU/mL was obtained by diluting bacterial cultures with PBS (pH 7.4). The bacterial suspension was added to bare CIP and CIP-NLC at a concentration ten times higher than MIC and placed in a 100 rpm shaking incubator at 37°C. Subsequently, 100 µL of each sample was collected and swabbed on MHA plates at predetermined intervals of 0, 1, 2, 4, 6, 12, and 24 h and plated in triplicate. The number of colonies was counted after incubation for 24 hours at 37°C.

2.7.3 Efflux pump inhibition

A previously reported cartwheel assay with modifications was used to perform an efflux pump inhibition study of CIP-NLCs against MRSA and *P. aeruginosa* efflux pumps using ethidium bromide (EtBr) [51]. The MHA containing 2 µg/mL of EtBr was prepared a day before the experiment and kept away from the light due to its light sensitivity of EtBr. The bacterial suspension of 1.5 x10^8 CFU/mL equilibrated at 0.5 McFarland standard was prepared from bacterial culture. The bacterial suspension was then seeded into a 96-well plate before treatment using bare CIP and CIP NLCs at subinhibitory concentrations (0.25 MIC) and untreated sample for a negative control to make the final volume of 200 µL. The plate was then incubated for one hour at 37°C. Subsequently, 100 µL was pipetted onto MHA containing EtBr and swabbed in a cartwheel before 24 hours of incubation. A UV transilluminator was used to visualize accumulated EtBr by the treated bacteria using a negative control as a reference.

2.7.4 Biofilm eradication studies

A microtiter plate assay quantified the elimination of biofilm formation by CIP-NLCs against MRSA and *P. aeruginosa* [52]. The bacterial culture was adjusted to 0.5 McFarland's standard before culturing in MHB (pH 7.4), and 100 µL was added into 96-well plates. The plates were incubated for 14 days at 37 °C. After incubation, plates were rinsed with PBS (pH 7.4) to remove non-adherent bacteria. Subsequently, biofilms were treated with 100 µL of CIP-NLCs and bare CIP at ten times MIC and incubated at 37 °C for 24 hours. Afterward, treatments and non-adherent bacteria were removed using PBS and dried for 15 minutes. The wells were stained with 0.1 % (w/v) crystal violet solution and incubated at 25 °C for 15 minutes before washing with PBS (7.4). It was then solubilized with 30 % acetic acid. The absorbance was determined using a Spectrostar Nano plate reader at 550 nm. The % biofilm eradication was quantified according to the following equation:

$$\%\text{Biofilm eradication} = \left(100 - \frac{A550\ nm\ treated\ cells}{A550\ nm\ untreated\ cells}\right) \times 100 \quad (5)$$

2.8 Antioxidant Activity Evaluation

The antioxidant activity of CIP-NLCs was assessed based on their DPPH scavenging properties using a previously reported method with manipulations [53]. The scavenging activities of CIP-NLCs, eugenol, and TS at concentrations ranging from 25 to 100 mg/mL were investigated using DPPH as stable radicals and ascorbic acid was used as a positive control. The CIP-NLCs, TS, eugenol, and ascorbic acid were mixed at a 1:3 volume ratio with a DPPH solution (180 µmol/L in methanol) using methanol as a negative control. Subsequently, test samples were incubated at 37°C away from light before their absorbances were measured at 517 nm using a microplate reader. The percentage of DPPH-radical scavenging of each sample was calculated according to the following equation:

$$\%\ \text{DPPH-radical scavenging} = \left(\frac{A0 - As}{A0}\right) \times 100 \quad (6)$$

Where *A0* is absorbance of the negative control, *As* is the absorbance of the sample.

2.9 *In vivo* antibacterial activity

In vivo antibacterial study of CIP-NLCs was performed following a mouse model of MRSA systemic infection method. All the animals were cared or handled following an approved protocol from the Institutional Ethics Review Committee (IERC) of the United States International University-Africa (USIU-A) (Approval number: USIU-A/IERC/FS93-2023). BALB/c mice weighing between 45-50 g were obtained. The mice were injected intraperitoneally with 200 µL of MRSA ($2.5x10^9$ CFU/mL) in PBS (7.4) to cause a systemic bacterial infection. Thereafter, mice were separated in three groups (n = 4) according to treatments that were going to be administered. After one hour post infection, the negative control group was treated with 500 µL of normal saline, the positive control and the test groups were treated with 500 µL of bare CIP and CIP-NLCs at 0.25 mg/mL, respectively. All the treatments were administered intraperitoneally. The mice were kept under observation for any signs of pain, sickness, or distress for 24 hours. After 24 hours post treatment, the mice were euthanized and sacrificed. The blood, liver, and kidney of the mice were tested for microbial burden. The blood was collected in a heparinized micro-tube and serial dilutions of the blood were plated on M17 agar plates. Then, the colonies were calculated after 24 hours of incubation at 37ºC. The livers and kidneys were aseptically harvested from each mouse and homogenised in sterilized in PBS (7.4). The homogenates were plated in triplicate in M17 agar plates. The colonies were then calculated after 24 hours of incubation at 37ºC. The following equation was used to calculate the CFU/mL:

$$\text{CFU/mL} = \frac{(number\ of\ colonies\ x\ dilution\ factor)}{volume\ of\ culture\ plate} \quad (7)$$

2.10 Statistical analysis

All experiments reported in this study were performed in triplicate, and data were summarised and presented as the mean ± standard deviation (SD). Statistical analysis for the data obtained from particle size, PDI, ZP, EE%, *in vitro* drug release, and *in vivo* antibacterial activity was carried out using one-way analysis of variance (ANOVA) with differences that were considered significant at a *P value* less than 0.05.

3. Results and discussions

3.1 Preparation and characterization of CIP-NLCs

Optimal physicochemical properties of nanoparticles provide an essential role in their behaviour at biological settings [54]. Particle size and zeta potential (ZP) are critical parameters to be investigated during the design of a nanoparticles which are responsible for their various biological effects, including dissolution [55], cellular uptake [56], and toxicity [57]. Preliminary screening was conducted using different parameters, including lipid ratio, surfactant types, and concentrations, to obtain an optimum formulation with preferable characteristics such as smallest size, narrow PDI, acceptable ZP, and highest entrapment efficiency (%).

3.1.1 Effect of lipid ratio on CIP-NLCs

The selection of suitable lipids and their ratios is highlighted as an essential requirement to achieve the desired bioactive outcomes in the design of NLCs. In this study, a potential tocopherol-to-eugenol (TS: eugenol) ratio to provide a more stable NLC formulation was determined and parameters such as surfactant type (Tween 20) and concentration, and sonication time remained fixed. In contrast, different ratios of lipids were varied. The Effect of lipid ratio on NLCs was evaluated using particle size, PDI, and ZP. With various lipid ratios, particle size ranged from 170 to 190 nm, PDI from 0.250 to 0.340, and ZP from -4.16 to -15.7 Mv. Nanoparticles with a negative ZP are reported to exhibit desired biological effects *in vivo* [88], and reduced ZP improves cell attachment and proliferation [59]. Negatively charged nanoparticles prevents electrostatic interaction which would result in non-selective targeting *in vivo*, as cell materials are predominantly negatively charged [60]. As can be seen in **Table 1**, the NLCs formulation with a lipid ratio of 1:2 showed the lowest particle size, followed by 1:1 and 2:1. However, despite the lowest particle size in the 1:2 formulation, theformulation with 1:1 lipid ratio had the narrowest PDI and most desired ZP compared to other formulations. Therefore, the 1:1 formulation, which had a particle size of 178.3 nm, PDI of 0.251, and ZP of -4.16 Mv, was further screened for other parameters to obtain an optimized CIP-NLCs formulation as presented in **Table 1:**

The effect of lipid ratio on CIP-NLCs. Results in the table are presented as mean ± SD (n = 3).

Lipid ratio (TS: eugenol)	Particle size (nm)	PDI	ZP (mV)
1:1	178.3 ± 3.041	0.251 ± 0.011	-4.16 ± 2.284
1:2	170.9 ± 3.87	0.433 ± 0.063	-15.7 ± 0.897
2:1	194.5 ± 1.750	0.317 ± 0.062	-22.2 ± 1.02

3.1.2 Effect of surfactants

The type and concentration of surfactant significantly affect the quality and stability of NLCs formulations [61]. The NLCs formulations with a lipid ratio of 1:1 was screened for a potential surfactant to achieve an NLCs formulation with the smallest particle size and narrow PDI using surfactants with varying molecular weights. In addition to the Tween 20 NLCs formulation prepared in 3.1.1, NLCs formulations containing Span 80 and Poloxamer 188 were prepared for screening of a suitable surfactant. All these surfactants were applied at a ratio of 1:1:0.5 (TS: eugenol: surfactant) and other parameters were constant. These different surfactants significantly affected the particle sizes and PDI of the NLCs, with particle sizes ranging from 178 to 586 nm and PDI from 0.251 to 0.633. Tween 20 showed the lowest particle size, followed by Poloxamer 188 and Span 80, as shown in **Table 2**. Therefore, Tween 20 was selected as the most suitable surfactant to fabricate more stable CIP-NLCs with a particle size of 178.3 nm and PDI of 0.251.

Table 2: The effect of surfactant type on NLCs. Results in the table are presented as mean ± SD (n = 3).

Surfactants	Particle size	PDI	ZP (mV)
Span 80	586 ± 59.59	0.633 ± 0.066	-7.07 ± 1.52
Tween 20	178.3 ± 3.041	0.251 ± 0.011	-4.16 ± 2.84
Poloxamer 188	357.80 ± 24.63	0.342 ± 0.110	-6.30 ± 1.85

After Tween 20 was chosen as the suitable surfactant, NLCs formulations with varying ratios (0.33, 0.67,1% m/v) of Tween 20 loaded with 10 mg of CIP were prepared. All other parameters were kept constant to investigate the effects of surfactant concentration on particle size, PDI, ZP, and EE %. As presented in **Table 3** the surfactant ratio increased from 1:1:0.5 to 1:1:1, the particle size decreased from 178.3 nm to 147.4nm, EE % increased from 80.96 % to 82.8 % and PDI and ZP did not show any significant change. However, increasing the surfactant ratio from 1:1:1 to 1:1:1.5 increased the particle size to 196.0 nm and PDI to 0.419, and decreased the EE % to 81.07 % indicating that high surfactant concentrations increase the particle size, PDI, and EE % of NLCs. Excessive use of surfactants in NLC formulations leads to their toxicity and agglomeration outcomes [62]. In addition,

Studies have demonstrated that overused surfactants can result in their self-assembly, decreasing the amount of surfactant available toimprove the interface quality of nanoparticles for stability [63].

The screening of different parameters of the NLCs showed that the optimum CIP-NLCs with particle size of147.4 ± 0.59 nm, PDI of 0.219 ± 0.009, ZP of -9.64 ± 2.22 mV, and 82.8 ± 0.39 %, were formulated using 1:1 (TS: eugenol) ratio and the concentration of Tween 20 at a ratio of 1:1:1. These optimum CIP-NLCs were used for further characterization.

Table 3: The effect of surfactant concentration on NLCs. Results in the table are presented as mean ± SD (n = 3).

Surfactant ratio (TS: eugenol: Tween 20)	**Particle size (nm)**	**PDI**	**ZP (mV)**	**EE %**
1:1:0.5	178.3 ± 3.041	0.251 ± 0.011	-4.16 ± 2.84	80.96 ± 0.57
1:1:1	147.4 ± 0.59	0.219 ± 0.009	-9.64 ± 2.22	82.8 ± 0.39
1:1:1.5	176.0 ± 1.026	0.419 ± 0.026	-7.80 ± 0.404	81.07 ± 1.59

3.1.3 Morphology of CIP-NLCs

The microscopic visualization of CIP-NLCs under TEM was used to confirm their size and morphology. The images (**Figure 1**) showed smooth spherical-shaped CIP-NLCs with an average diameter of 122.64 ±5.71 nm (n=3) and homogeneous size distribution. These size measurements are slightly lower than that obtained in DLS. The DLS instrument measures hydrodynamic radius, which only provides a hypothetical measurement, while TEM measures the actual particle size [64, 65].

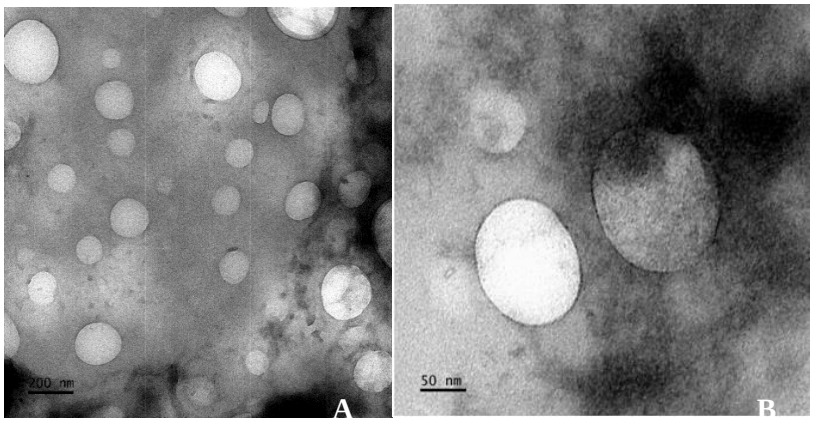


Figure 1: TEM image of CIP-NLCs A (200 nm) and B (50 nm)

3.2 *In vitro* CIP-NLCs biosafety studies

3.2.1 Hemocompatibility

The compatibility of nanocarriers to red blood cells remains the main requirement for any desired nano

drug delivery systems with the potential to improve the therapeutic index of several antibiotics. The hemocompatibility study of nanocarriers is based on maintaining the upregulated haemoglobin levels at systemic levels after treatment with novel nanocarriers [66]. In this study, CIP-NLCs showed non-haemolytic activity in the red blood cells at concentrations ranging from 0.05 mg/mL to 0.5 mg/mL as the haemolysis of 0.3 mg/mL is less than 2% as illustrated in **Figure 2**, which is within the negligible haemolysis of less than 5% [67]. Therefore, these results indicate that CIP-NLCs may be suitable for parenteral administration.

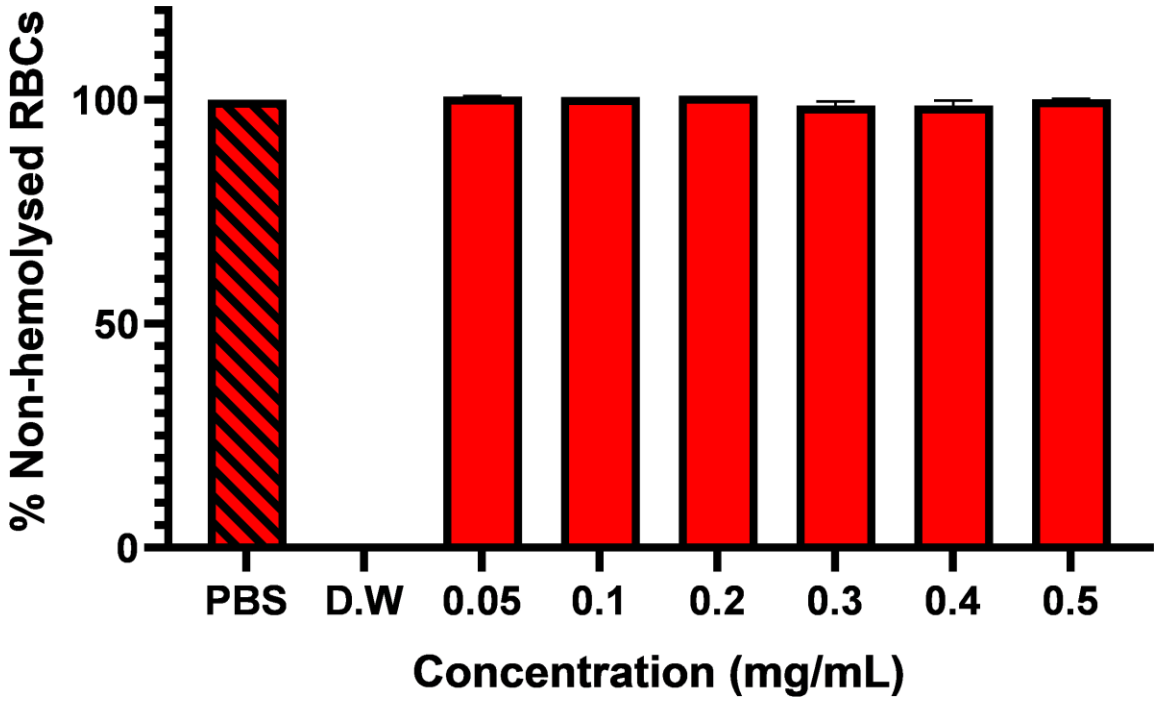


Figure 2: Haemolytic activity of CIP-NLCs, distilled water (negative control), and PBS (positive control) at different concentrations against red blood cells. Data in the graphs are presented as mean ± SD (n = 3).

3.3 *In vitro* drug release and kinetics studies

The drug release profiles of CIP-NLCs were compared to that of bare CIP for 48 hours at 37 °C using the dialysis bag diffusion technique, with results in **Figure 3**. The release of bare CIP was completed within four hours. Meanwhile, the release of CIP from CIP-NLCs demonstrated biphasic behaviour, which exhibited a burst release of CIP for the first four hours. After six hours, sustained release behaviour of CIP-NLCs was observed, where only 67% of CIP was released after 48 hours of incubation. CIP-NLCs showed a sustained release profile compared to bare CIP. A similar release trend was previously reported in the study conducted by Muraca *et al*. (2021) [68] on the delivery of CIP

using NLCs. The initial burst release of CIP-NLCs might be due to the drug adsorbed on the surfaces of NLCs, while the slower release of CIP might be due to the highly ordered structure of the lipid matrix of the NLCs retaining the release of CIP [69].

Developing a nanosystem with sustained drug release requires the proper understanding of release kinetics. The cumulative drug release data of CIP-NLCs over time were further fitted to various release kinetic models, and the parameters were subsequently calculated. The highest R^2 value was 0.8901, while the lowest RMSE value was 5,0693. Consequently, the Weibull model was thought to be the best-fit model for the release of the CIP-NLCs. Several studies have documented the Weibull model as adequate to explains the drug release patterns from various types of nanoparticles [70]. The Weibull release exponent determines the mechanism of drug release (β) value, $\beta \leq 0.75$ suggesting a Fickian diffusion, and $0.75 < \beta < 1$ indicating a hybrid mechanism, whereas a $\beta > 1$ indicates a collapse mechanism. The β value of CIP-NLCs is 0.171, demonstrating that Fickian diffusion is the proposed release mechanism. These findings were consistent with the Korsmeyer-Peppas equation (n) value of 0.134, suggesting Fickian diffusion as $n \leq 0.43$.

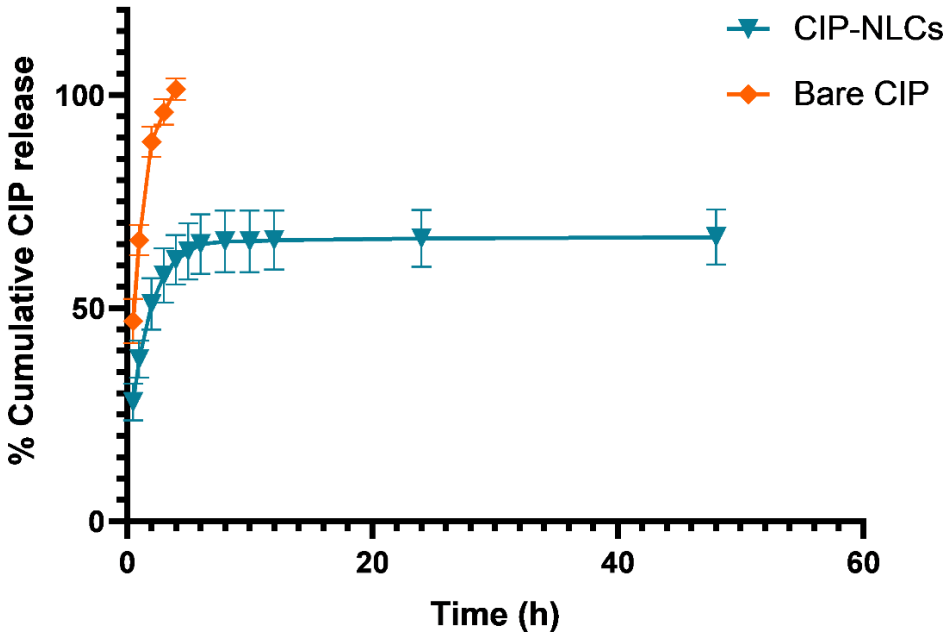


Figure 3: *In vitro* drug release of bare CIP and CIP-NLCs at pH 7.4 for 48 hours. Data in the graph are presented as mean ± SD (n = 3).

Table 4: Drug release kinetics data for CIP-NLCs

Model	R^2	RMSE	Rsqr_adj	AIC	MSC	Release exponent
Zero-order	12.36	42.08	-11.3800	121.9809	-2.6795	-
First order	-0.7022	18.04	-1.6590	103.0517	-1.1021	-
Higuchi	-2.6691	24.2546	-3.1902	108.9682	-1.5951	-
Hixson-Crowell	-3.8854	26.2672	-3.7894	110.5880	-1.7301	-
Korsmeyer-Peppas	0.6805	6.9810	0.6515	80.0318	0.8162	0.134
Weibull	0.8901	5.0693	0.8643	69.4470	1.6983	0.171

R^2=linear regression coefficient
RMSE=Root mean square error
Rsqr=adjusted coefficient of determination
AIC=Akaike Information Criterion
MSC=Model Selection Creterion

3.4 *In vitro* antibacterial activity studies

3.4.1 *In vitro* antibacterial and FIC index

The micro broth dilution method was used to determine the antibacterial activity of CIP-NLCs, free CIP, and blank-NLCs tested against MRSA, SA, *P. aeruginosa,* and *E. Coli*. As displayed in **Table 5**, the MIC values of bare CIP against SA and MRSA were 0.39 μg/mL and 25 μg/mL, respectively. MIC value of CIP-NLCs against SA was 0.195 μg/mL, whereas MIC values of CIP-NLCs against MRSA were 12.5 μg/mL and 25 μg/mL. These results indicate a 2-fold reduction in the MIC values of CIP-NLCs against both SA and MRSA. The antibacterial activity of CIP-NLCs extended up to 72 hours, while bare CIP showed no activity after 48 hours. Furthermore, the MIC values for bare CIP against *P. aeruginosa* and *E. coli* were 0.39 μg/mL and 0.195 μg/mL, respectively. the MIC value of CIP-NLCs against *P. aeruginosa* was 0.097 μg/mL and 0.195 μg/mL, while the MIC values of CIP-NLCs against E. *coli* were 0.0248 μg/mL and 0.0485 μg/mL. The antibacterial activity of CIP-NLCs against *P. aeruginosa* and *E. coli* showed 4-fold reduction of the bacteria compared to bare CIP. It is observed from these results that CIP has better antibacterial activity against *P. aeruginosa* and *E. coli* than MRSA. CIP prevents the bacterial DNA replication through the inhibition of DNA topoisomerase and DNA gyrase which are located in the nucleus [31]. The enhanced antibacterial activity of CIP-NLCs when compared to bare CIP, could be attributed to their reduced size with large surface area as well as their hydrophobic nature, which could have improved their interaction and uptake of the cell wall of the bacteria., therefore improving CIP activity [71]. In addition, the antibacterial activity of CIP-NLCs might be due to the structural difference of the cell wall in target bacteria (gram-positive or gram-negative). The thick peptidoglycan in the cell wall in gram- positive bacteria forms a barrier for partitioning of CIP to reach the site of action [72]. Therefore, as can be seen in **Table 5**, CIP-NLCs and bare CIP are hindered by the structure of gram-positive bacteria. Hence, CIP-NLCs and bare CIP

demonstrated a notable difference in antibacterial activity (presented in **Table 6**) when compared to gram-positive bacteria.

As displayed in **Tables 5** and **6**, the antibacterial activity of CIP-NLCs against all tested bacteria strains extended for up to 72 hours. Conversely, bare CIP against MRSA showed antibacterial activity for 48 hours at the given concentrations. In contrast, against *P. aeruginosa*, CIP loses activity over time as the MIC values increase at different time intervals. The extended antibacterial activity of CIP-NLCs is attributed to the sustained drug release of CIP from the NLCs [73, 42]. In addition, MIC values of CIP-NLCs against MRSA increased from 12.5 µg/mL to 25 µg/mL from 24 to 48 hours, respectively. MIC values of CIP-NLCs against *P. aeruginosa* increased from 0.097 µg/mL to 0.195 µg/mL from 24 to 48 hours, respectively. The fast (initial burst) release of CIP from the NLCs before reaching the sustained drug release might have been attributed to this antibacterial activity in the first 24 hours. The sustained release profile of CIP from CIP-NLCs is ideal for parenteral administration, as it is reported to prevent potential co-infection [74].

The Σ FIC at each time interval, as shown in **Table 7,** was calculated for further understanding of the combined antibacterial Effect of blank-NLCs and CIP in the CIP-NLCs against *E. coli* and *P. aeruginosa*. The Σ FIC values for 24, 48, and 72 hours against *E. coli* were all found to be 0.25, indicating a synergistic antibacterial activity. In the same way, the Σ FIC values against *P. aeruginosa* were 0.25, 0.25, and 0.125, respectively, also indicating a synergistic effect. These Σ FIC indices could be due to eugenol, which is synergistic when combined with antibiotics [34].

Table 5: Antibacterial activity of CIP-NLCs on gram-positive bacteria (n = 3).

Time (h)	**24**	**48**	**72**	**24**	**48**	**72**
			MICs (µg/mL)			
Bacteria	SA			MRSA		
Bare CIP	0.39	0.39	0.39	25	50	NA
CIP-NLCs	0.195	0.195	0.195	12.5	25	25
Blank-NLCs	NA	NA	NA	NA	NA	NA

Table 6: Antibacterial activity of CIP-NLCs on Gram-negative bacteria (n = 3)

Time (h)	**24**	**48**	**72**	**24**	**48**	**72**
			MICs (µg/ml)			
Bacteria	*E. coli*			*P. aeruginosa*		
Bare CIP	0.097	0.097	0.195	0.39	0.78	1.56
CIP-NLCs	0.0243	0.0243	0.0485	0.097	0.195	0.195
Blank-NLCs	103,125	206,25	206,25	412,5	412,5	412,5

NA=No activity*

Table 7: ΣFIC values against *E. coli* and *P. aeruginosa* at each time interval

Time (h)	*E. coli*		*P. aeruginosa*	
	ΣFIC	**Interpretation**	**ΣFIC**	**Interpretation**
24	0.25	Synergism	0.25	Synergism
48	0.25	Synergism	0.25	Synergism
72	0.25	Synergism	0.125	Synergism

3.4.2 Times Killing assay.

To further analyse antibacterial activity, the rapidity of CIP-NLCs to eliminate MRSA and *P. aeruginosa* was investigated. The results of the time-killing analysis of CIP-NLCs and bare CIP at a concentration five times increased MIC against MRSA are illustrated in **Figure 4A**. After 8 hours of incubation, bare CIP eliminated 85 % of MRSA, whereas CIP-NLCs eliminated 100 % of bacteria (approximately 5 log reduction). After incubation for 24 hours, bare CIP exhibited approximately 5 log reduction. Hence, CIP-NLCs could be administered to eliminate MRSA infections effectively faster than conventional CIP.

Furthermore, **Figure 4B** represents the rate at which CIP-NLCs and bare CIP exert their bactericidal activity against *P. aeruginosa*. After 1 hour of incubation, CIP-NLCs eliminated 100 % of *P. aeruginosa* populations, which is more than 6 log reduction. On the other hand, bare CIP eradicated 100% bacteria (≥ 6 log reduction) after 24 hours of incubation. Thus, CIP-NLCs could rapidly treat infections associated with *P. aeruginosa*.

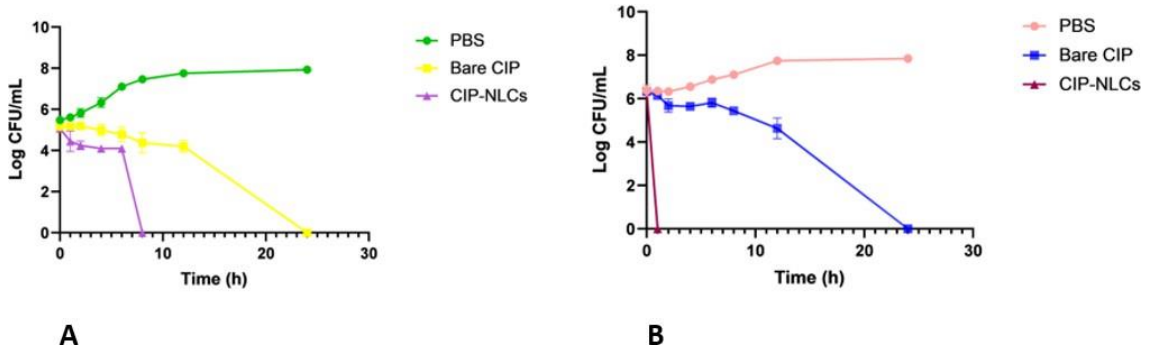


Figure 4: Killing kinetics of MRSA (**A**) and *P. aeruginosa* (**B**) exposed to bare CIP and CIP-NLCs at 5X MIC and PBS pH 7.4 (control). Data in the graphs are presented as mean ± SD (n = 3).

3.4.3 Efflux pump inhibition

Efflux transporters expel a wide range of noxious agents that penetrate the bacterial cell wall, including antibiotics, detergents, and dyes [75]. An efflux pump inhibition study was conducted on a cartwheel assay, which is based on the accumulation of ethidium bromide dye and thereby induces fluorescence [50]. Cartwheel assay compared the efflux pump inhibition of CIP-NLCs and bare CIP, and the untreated bacterial suspension was set as a negative control. In addition, anti-efflux adjuvants such as TS and eugenol were investigated against MRSA and *P. aeruginosa*, respectively. In this study, the accumulation of ethidium bromide by the treated cells was indicated by fluorescence visualised under a UV-transilluminator instrument.

NorA and *NorB* are widespread chromosomally encoded efflux pumps contributing to multidrug resistance, rendering gram-positive bacteria resistant to a wide range of substrates, including antibiotics [76]. As can be seen in **Figure 5B**, CIP- NLCs showed the highest fluorescence intensity compared to all the samples tested against MRSA. This notable fluorescence might be due to the inhibition of MRSA efflux pumps, which would then allow the accumulation of EtBr. Additionally, TS showed a high fluorescence when investigated against MRSA. These results corroborated the findings of a study reported by Salih *et al*. (2020) [33], which demonstrated an increased potential of TS towards the inhibition of *NorA* and *NorB*. Therefore, CIP-NLCs can be used as potent inhibitors of gram-positive efflux pumps.

Similarly, CIP-NLCs were investigated for their efflux pump inhibition against gram-negative bacteria (*P. aeruginosa)*. *MexA* and *AcrA* are predominant efflux pumps in gram-negative bacteria, which mediate multidrug resistance and play a significant role in the pathogenicity of this bacteria [77]. The high fluorescence of *P. aeruginosa* that was treated with CIP-NLCs could be due to inhibition of efflux pumps, which would subsequently enable the accumulation of EtBr. Eugenol was investigated for its efflux pump inhibitory capabilities against *P. aeruginosa.* Eugenol showed high fluorescence intensity against *P. aeruginosa* which might be due to the inhibition of efflux pumps. The findings confirmed eugenol's documented efflux pump inhibition against gram-negative efflux pumps [35]. Therefore, the potential of CIP-NLCs to inhibit gram-negative efflux pumps might be due to eugenol.

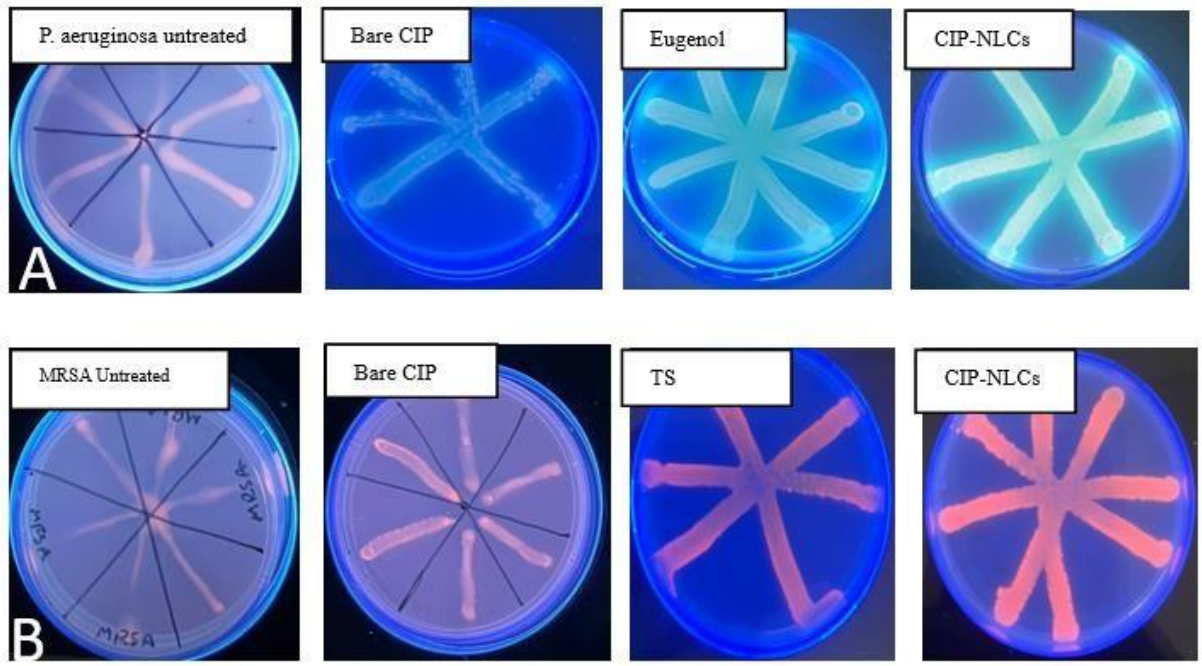


Figure 5: Efflux pump inhibition of CIP-NLCs and bare CIP compared to eugenol and TS (0.25 X MIC) against *P.aeruginosa* (**A**) and MRSA (**B**), respectively.

3.4.4 Biofilm eradication studies

Biofilms represent an additional strategy by which bacteria survive sublethal antibiotic concentrations and further increase antimicrobial resistance. This bacterial behaviour has been identified as a primary contributing factor to the significantly growing antibiotic resistance, as biofilms protect from antibiotics and host immunity [78]. The effective eradication of biofilm using the conventional dosage forms of antibiotics would necessitate higher and more frequent doses, resulting in the increased risk of dose-dependent toxicity. The efficacy of CIP-NLCs to reduce the biofilm mass was investigated against MRSA and *P. aeruginosa* using crystal violet at concentrations ten times higher than the respective MICs. Nanosized antibiotic delivery systems have been reported to effectively penetrate the extracellular polysaccharides of biofilms. CIP-NLCs eradicated 57% of MRSA biofilms, which is approximately 3-fold higher than the biofilm eradication of bare CIP (*P*-value <0.00136), as illustrated in **Figure 6A**.

In contrast, CIP-NLCs have eliminated 56 % of *P. aeruginosa* biofilms, which is a 1.25-fold increase compared to bare CIP (*P*-value <0.0195), which was not as significant as MRSA biofilm eradication. These results have demonstrated the increased activity of the conventional CIP in the elimination of *P. aeruginosa* biofilm. The superior biofilm eradication of CIP-NLCs might be due to the negative zeta potential of these nanocarriers, which facilitates the binding to the positively charged structure of the biofilms [79]. The remarkable elimination of MRSA biofilm mass using CIP- NLCs was improved by TS, which is reported to decrease the adhesion of gram-positive bacteria to the biofilm matrix while increasing their exposure to the antibiotics loaded in nanocarriers [80]. Furthermore, eugenol is

reported to effectively inhibit the biofilms from gram-negative bacteria [81]. Therefore, nanoformulations, including tocopherol succinate and eugenol, would notably decrease the considerable risk associated with using indwelling instruments during different therapeutic procedures.

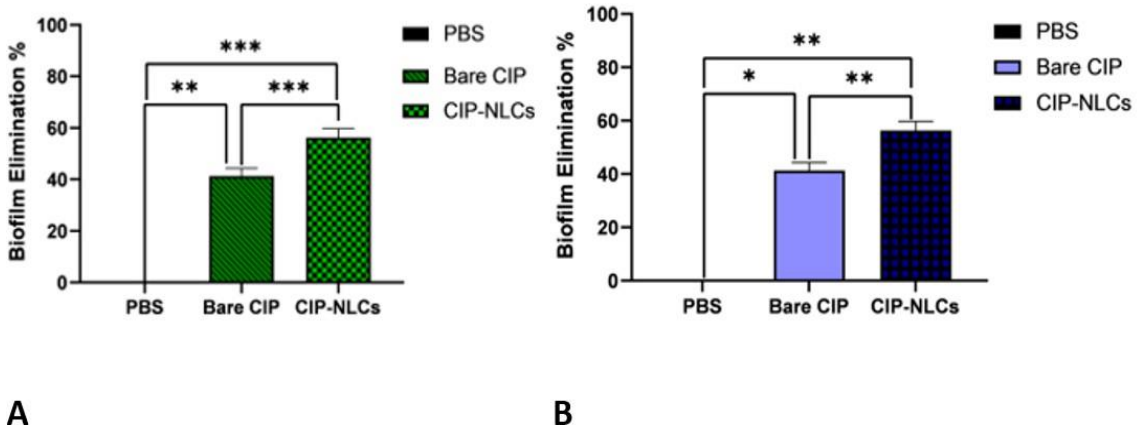


Figure 6: Percentage of MRSA (**A**) and *P. aeruginosa* (**B**) biofilms after treatment with bare CIP, CIP-NLCs, and PBS pH 7.4 (control). CIP-NLCs showed significant improvement in the inhibition against both strains (*P*- values * >0.05, ** >0.01, and *** >0.0001). Data in the graphs are presented as mean ± SD (n = 3).

3.5 *In vitro* antioxidant activity

Oxidative stress is defined as an excessive production of free radicals causing an imbalance between free radicals and antioxidants in the body. The excessively produced free radicals negatively affect several biological components, such as structural damage of DNA. Consequently, these impaired DNA are critical pathogenesis of several diseases [82]. Although produced in insufficient amounts in the body, antioxidants play a key role in maintaining these free radicals. Natural exogenous antioxidants such as phenols and vitamin C and tocopherols compensate for this shortage. The antioxidant activity is associated with a beneficial effect on immune function as they restore cell-mediated immunity during infection [83]. Therefore, nanocarriers with antioxidant for the delivery of antibiotics would provide them with alternative mechanisms of action.

The present work investigated the DPPH radical scavenging activity of CIP-NLCs, ascribed from the reported potential antioxidant behaviour of eugenol and TS. Ascorbic acid was treated as a positive control for this study as it is reported to have a superior antioxidant activity [84]. CIP-NLCs demonstrated excellent antioxidant activity comparable to that of ascorbic acid. The percentage of DPPH inhibited by CIP-NLCs at 100 µg/mL was 90 %, whereas the ascorbic acid standard inhibited 91 % of DPPH. However, at 25 µg/mL, CIP-NLCs showed increased free radical scavenging compared to the positive control. These results are due to the presence of eugenol, which showed similar DPPH scavenging to ascorbic acid, and TS maintained the increased antioxidant activity of

CIP-NLCs in all concentrations. Therefore, the most notable antioxidant activity of these CIP-NLCs could be exploited to maintain the free radicals at low concentrations and prevent disease pathogenesis.

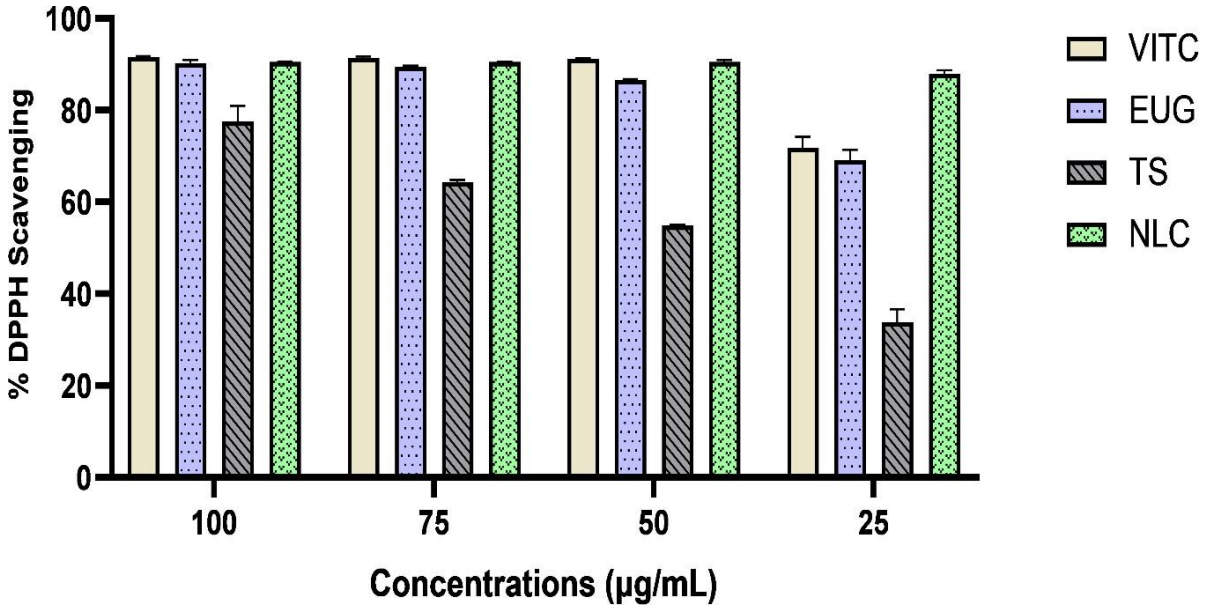


Figure 7: Percentage of DPPH radical scavenging of CIP-NLCs, eugenol, TS, and Ascorbic acid (control).

3.6 *In vivo* antibacterial activity

The *in vitro* antibacterial studies of CIP-NLCs revealed their superiority in eradication of MRSA bacteria compared to bare CIP. This concept was further investigated using *in vivo* against a mouse (BALB/c) model of MRSA systemic infection. After classifying the mice into negative, positive, and treatment groups, the MRSA infected mice were treated with normal saline, bare CIP, and CIP-NLCs, respectively. The mice were euthanized after 24 hours of infection and treatment, and the CFU/mL was determined from each group from blood, liver, and kidney.

The comparison of CFUs recovered from the blood which is illustrated in **Figure 8C** showed that normal saline, bare CIP, and CIP-NLCs groups had CFU counts of 1.075×10^9, 1.85×10^8, and 666,667 CFU/mL, respectively. These counts represented a 1613-fold and 5.81-fold MRSA reduction of CIP-NLCs and bare CIP, respectively, when compared to normal saline (negative control) group. These results indicated a significant elimination of MRSA infections in mice treated with bare CIP and CIP-NLCs ($p < 0.0001$). Additionally, CFUs recovery difference between CIP-NLCs and bare CIP treatment groups was further computed. This comparison represented a 277.50-fold better activity of the nanocarrier compared to bare CIP which was not significant ($p = 0.1617$).

As shown in **Figure 8A**, CFUs recovered from the liver were 1.74×10^8, 8.23×10^7, and 3.9×10^7

CFU/mL for normal saline, bare CIP, and CIP-NLCs groups, respectively. These recovered CFUs of bare CIP and CIP-NLCs were 2.12-fold ($p = 0.0523$) and 4.47-fold ($p = 0.0102$) reduction of MRSA over normal saline, respectively. The results indicate a significant reduction of the MRSA burden from the group that was treated by CIP-NLCs. Furthermore, CFUs recovered from CIP-NLCs, and bare CIP treatment groups were compared. This comparison represents a not significant reduction of MRSA burden from the liver ($p = 0.3862$) with 2.11-fold lower than bare CIP.

In addition to the above, CFUs recovery from the kidney injected with normal saline, bare CIP, and CIP-NLCs were 1.51×10^8, 6.77×10^7, and 1.23×10^7 CFU/mL, respectively, as illustrated in **Figure 8B**. These findings represent a 2.24-fold ($p = 0.0254$) and 12.27-fold ($p = 0.0023$) significant reduction of MRSA burden in kidneys treated with bare CIP and CIP-NLCs, respectively. A further comparison of CFU recovery of CIP-NLCs confirmed a 5.49 reduction when compared to bare CIP. This result represented a non-significant reduction of MRSA from the kidney ($p = 1157$).

The *in vivo* antibacterial activity of CIP-NLCs showed a significant reduction of MRSA infections in mice compared to bare CIP. Although CIP-NLCs significantly eradicated MRSA over bare CIP in the liver, it had better activity against the MRSA that was colonizing the blood and the kidneys. The reduced activity against MRSA infections in the liver might be due the lack of materials such as polyethylene glycol that might have provided protection to CIP-NLCs against the uptake by reticuloendothelial system [85]. The maximum MRSA reduction was notable in blood collected from the group that was treated with CIP-NLCs. These results are consistent with the findings of the study conducted by Liao et al., (2021) [86] who evaluated the ability of NLCs for the co-delivery of CIP and rolipram for MRSA.

These results confirmed the superior antibacterial activities of CIP-NLCs against MRSA infections over the conventional CIP. The enhanced anti-MRSA of these nanocarriers may ascribe from several reasons. The reduced particle size, large surface area and lipophilicity of the NLCs which maximize their interaction with bacterial cell wall and cellular uptake [42]. Moreover, the increased elimination of MRSA might be due to the efflux pump inhibitory effect of CIP-NLCs. The inhibition of these efflux pumps could improve the intracellular concentration of CIP in the bacterial infection site.

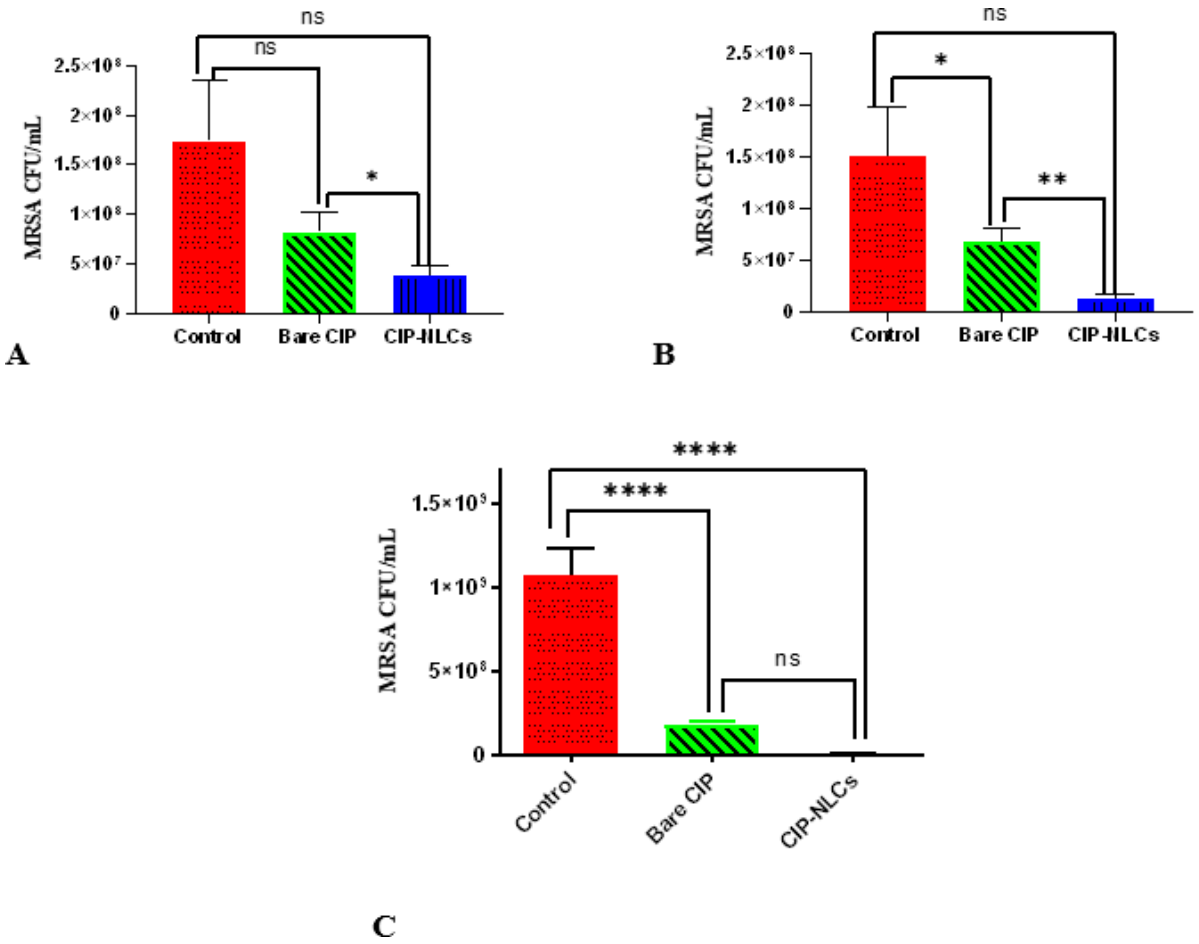


Figure 8: Quantification of MRSA $\log_{10}$ CFU/mL in BALB/c mice after 24 hours of treatment with control, bare CIP, and CIP-NLCs. (**A**) Liver, (**B**) Kidney and (**C**) Blood. Data is presented as mean ± SD (n = 3). (ns= not significant, p values ** = < 0.01, *** = < 0.001, **** = < 0.0001).

4. Conclusion

The recent popularity of nano drug delivery systems has broadened research prospects into exploring the different mechanisms bacteria use to tolerate lethal concentrations of conventional antibiotics. Thus, the current study aimed to formulate and assess CIP-NLCs that would inhibit the overexpression of efflux pumps to achieve sufficient antibiotic concentrations at bacterial infection sites. Antioxidant lipids with reported efflux pump inhibition were used to prepare CIP-NLCs. The NLCs were designed using the well-documented hot homogenization/ultrasonication technique and were optimized to obtain CIP-NLCs with acceptable particle size, PDI, ZP, and EE%. The *in vitro* biocompatibility evaluation results showcased CIP-NLCs as biosafe for further *in vitro* investigations. The release of CIP from CIP-NLCs at pH 7.4 showed a biphasic release profile with an initial burst release followed by sustained release. The *in vitro* antibacterial studies of CIP-NLCs against SA, MRSA, *E. coli*, and *P. aeruginosa* revealed their superior antibacterial efficacy compared to the conventional dosage of CIP. Bacterial kinetics of MRSA and *P. aeruginosa* showed a total bacterial clearance at 8 hours and 1 hour,

respectively. The cartwheel assay confirmed the efflux pump inhibitory activities of CIP-NLCs against MRSA and *P. aeruginosa*. The biofilm reduction of CIP-NLCs was superior to that of bare CIP when exposed to MRSA and *P. aeruginosa*. CIP-NLCs presented excellent DPPH free radical scavenging capabilities, further improving the treatment of bacterial diseases. Furthermore, the enhanced activity of CIP-NLCs against MRSA was also confirmed by the *in vivo* antibacterial studies. Therefore, the findings of this study confirmed the potential of CIP-NLCs as the efflux pump inhibitor with improved antibacterial efficacy and antioxidant properties and enhanced biosafety profile. Hence, CIP-NLCs could serve as a multi-functional antibiotic nanocarrier, which could overcome therapeutic limitations and, ultimately, antibiotic resistance.

Ethical statement

Animal studies were conducted according to the protocol approved by the Institutional Ethics Review Committee (IERC) of the United States International University-Africa (USIU-A) (Approval number: USIU-A/IERC/FS93-2023).

Author contributions

Sbongumusa Dlamini: Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing-original draft and editing. **Eman H. Elhassan**: Methodology, formal analysis, and editing. **Eman I. Abdalla**: Methodology and formal analysis. **Mohammed A. Gafar:** Methodology. **Xylia Peters:** Formal analysis and editing**. Calvin A. Omolo**: conceptualization, co-supervision, problem-solving, validation, and editing. **Thirumala Govender**: conceptualization, funding acquisition, supervision, problem-solving, validation, and editing.

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Conflicts of interest

The authors report no conflicts of interest in this work.

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Chapter 4 General conclusions

4.1 Introduction

Infectious diseases caused by bacterial infections are one of the common pathways leading to mortality or morbidity. The successful treatment of bacterial infections over the recent years was achieved using conventional dosage forms of antibiotics. However, the rampant use along with the well-documented limitations of these dosage forms has led to the growing antibiotic resistance crisis. Bacterial infections caused by MRSA and *P. aeruginosa* are highlighted as a major global public health concern due to their ability to develop resistance to a wide range of antibiotics (multidrug resistance). In addition, the bacteria are continuously developing mechanisms to withstand lethal concentrations of antibiotics such as efflux pumps and biofilms. Researchers are focusing on the development of novel strategies to counteract these challenges in an attempt to resuscitate antibiotic efficacy. Consequently, nanotechnology is being extensively explored for better delivery of antibiotic. Although nano antibiotic delivery has shown enhanced antibiotic therapy, there is still a need to optimize nanocarriers with better potential of overcoming antimicrobial resistance. Thus, exploitation of bacterial behavioural mechanisms has gained traction over the recent years. Efflux pump inhibitory nanocarriers are gaining interest owing to potential of the inhibiting efflux genes which are commonly associated with multidrug resistance. Nanostructured lipid carriers (NLCs) are efficient nanosystems for antibiotic delivery and superior antibiotic therapy. Therefore, the current research aimed to explore the potential of ciprofloxacin-loaded nanostructured lipid carriers (CIP-NLCs) which were designed from TS and eugenol for enhancing antimicrobial activity and overcoming resistance mechanisms against MRSA and *P. aeruginosa*.

The main conclusions generated from the research data are summarised below:

- The optimized formula of CIP-NLCs displayed a spherical shape with a size of 147.4 ± 0.59 nm, PDI of 0.219 ± 0.009, ZP of -9.64 ± 2.22 mV, and EE % of 82.8 ± 0.39%.
- The *in vitro* biosafety studies performed by haemolysis test revealed the safety of CIP- NLCs towards red blood cells.
- *In vitro* drug release studies showed a faster release of bare CIP, which was completelyreleased at four hours. CIP-NLCs demonstrated a biphasic release profile, where an initial burst release was observed followed by a sustained release.
- The *in vitro* antibacterial studies confirmed superiority of CIP-NCLs over bare CIP andblank-NLCs against SA, MRSA, *E. coli*, and *P. aeruginosa.* CIP-NLCs had a 2-fold lower MIC against SA and MRSA compared to bare CIP, whereas a 4-fold reduced MIC against *E. coli* and P. *aeruginosa* was observed. Furthermore, the sustained antibacterial activity of CIP-NLCs was extended up to three days, whereas bare CIP gradually lost activity over this period. The bacterial kinetic test of CIP-NLCs against MRSA showed complete eradication of the bacteria after eight hours of incubation, whereas bare CIP was completely eradicated after 24 hours. Additionally, the bacterial kinetics test of CIP-NLCs against *P. aeruginosa* revealed total elimination of the bacteria within one hour incubation, whereas bare CIP eliminated 100% of the bacteria after eight hours. The microtiter plate assay revealed 3- and 2-fold higher MRSA and *P. aeruginosa* biofilm elimination of CIP-NLCs when compared to bare CIP, respectively.
- The cartwheel assay confirmed the efflux pump inhibition of CIP-NLCs against efflux pumps associated with MRSA and *P. aeruginosa.*
- DPPH scavenging assay revealed the excellent antioxidant activity of CIP-NLCs that was comparable to Vitamin C which is a well-reported potent antioxidant.
- The *in vivo* systemic infection in BALB/c mice model was used to evaluate the efficacyof the CIP-NLCs. The results showed a 1613-fold, 4.47-fold, and 12.27-fold reduction of blood, liver, and kidney MRSA infection, respectively, that were treated with CIP- NLCs compared to the negative control and bare CIP.

4.2 Significance of the findings in the study

CIP-NLCs were successfully designed from TS and eugenol, and inhibited efflux pumps and enhanced the overall antibiotic therapy of CIP to overcome antibiotic resistance. The significance of the outcomes of the study includes the following:

New pharmaceutical products

This study has introduced novel pharmaceutical products, namely, CIP-NLCs with efflux pump inhibitory activities. CIP-NLCs have demonstrated a potential for administration using intravenous and intraperitoneal routes. The introduction of efflux pump inhibitory nanocarriers could widen the availability of advanced pharmaceutical adjuvants with the potential of targeting different mechanisms

of bacteria for the preparation of the effective antibiotics; and thereby, encouraging pharmaceutical industries to develop new antibiotics with an improved potential of treating bacterial infections and overcome antibiotic resistance.

Improved treatment of bacterial infections

The *in vitro* antibacterial activity studies of the newly formulated CIP-NLCs showed superior activity and sustained release of CIP for an extended period. This novel nanocarrier has the potential to enhance the treatment of bacterial infections in different ways including inhibition of efflux pumps which would increase the active drug concentration in bacterial cells and improvement of the overall therapeutic outcomes of the incorporated antibiotics which could be vancomycin, tetracycline, gentamycin, among others. Inhibition of efflux pumps would have a notable effect on overcoming antibiotic resistance as they can extrude a wide range of antibiotics. This promising approach would improve the availability of effective antibacterial therapies to improve the treatment of bacterial infections, save lives, and also improve the quality of lives.

Stimulation of new research

The successful development of CIP-NLCs and the results from the different experimental studies can stimulate novel research ideas, including:

- Due to the ability of the newly developed CIP-NLCs to target efflux genes from different resistant-bacteria, this strategy could be employed in the formulation of other efficient nanocarriers such as niosomes to combat efflux pump-mediated multidrug resistance.
- The excellent antioxidant activity of CIP-NLCs due to eugenol and TS could stimulate the research into the identification of other materials with antioxidant activities such as phenols. These materials would then be employed into the formulation of nanocarriers with the potential of balancing the free radicals and antioxidants, and thereby prevent oxidative stress which is a key requirement for pathogenesis in numerous diseases.
- The notable biofilm eradication of CIP-NLCs could stimulate research into evaluating the potential of TS and eugenol to interfere with bacterial signalling pathways for antibiofilm activity.
- These findings can provide research advancements to explore other natural compound such as trans-cinnamaldehyde for their application in the formulation of nanocarriers that inhibit other survival mechanisms of the bacteria.

5.3 Recommendations for future studies

This study highlighted the advantages of fabricating nanocarriers to inhibit additional survival strategies of the bacteria. In addition, *in vitro* studies confirmed the superior properties of CIP-NLCs over bare CIP in the treatment of bacterial infections. However, additional studies are still required to strengthen

the novelty and improve the delivery of CIP via CIP-NLCs.

The following studies are therefore recommended:

- Cytotoxicity studies should be conducted to prove the biosafety of CIP-NLCs to normal body cells.
- Computational outlook of CIP-NLCs and efflux pumps could enhance the understanding of their interactions.
- Real-time PCR studies should be performed to validate the inhibited efflux genes in a cartwheel assay.
- Simultaneous delivery of several antibiotics with different mechanisms of action from these NLCs can be explored to maximize therapeutic activities.
- Further *in vivo* studies including the drug pharmacokinetics and pharmacodynamics studies should be carried out to obtain more information regarding redox-responsive drug release, drug bioavailability, and drug biodistribution profiles.
- Short- and longer-term *in vivo* toxicity studies may also be carried out to obtain a thorough biosafety profile of the formulation.
- Stability studies in accordance with the international conference of harmonisation (ICH) for different regions should be conducted to ascertain the shelf-life of the formulation.

5.4 Conclusions

The findings of this study confirmed the potential of efflux pump inhibitory CIP-NLCs to improve antibacterial activity, overcome antibiotic resistance and may contribute to superior treatment of bacterial infections. This study has contributed to the advancement of the nanocarriers' strategies to overcome the drawbacks of conventional dosage forms of antibiotics and antibiotic resistance.

Appendix: Turnitin report

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Eman A. Ismail, Calvin A. Omolo, Mohammed A. Gafar, Rene Khan, Vincent O. Nyandoro, Elliasu Y. Salifu, Thirumala Govender. "Multi-functional pH-responsive and biomimetic chitosan-based nanoplexes for targeted delivery of ciprofloxacin against bacterial sepsis", International Journal of Biological Macromolecules, 2024 Publication	1%
2	Joshua C. Nwabuife, Calvin A. Omolo, Thirumala Govender. "Nano delivery systems to the rescue of ciprofloxacin against resistant bacteria “E. coli; P. aeruginosa; Saureus; and MRSA” and their infections", Journal of Controlled Release, 2022 Publication	<1%
3	Nawras Osman, Calvin A. Omolo, Ramesh Gannimani, Ayman Y. Waddad et al. "Novel fatty acid-based pH-responsive nanostructured lipid carriers for enhancing antibacterial delivery", Journal of Drug Delivery Science and Technology, 2019	<1%

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4	Van-An Duong, Thi-Thao-Linh Nguyen, Han-Joo Maeng. "Preparation of Solid Lipid Nanoparticles and Nanostructured Lipid Carriers for Drug Delivery and the Effects of Preparation Parameters of Solvent Injection Method", Molecules, 2020 Publication	<1%
5	Yajna Jaglal, Nawras Osman, Calvin A. Omolo, Chunderika Mocktar, Nikita Devnarain, Thirumala Govender. "Formulation of pH-responsive lipid-polymer hybrid nanoparticles for co-delivery and enhancement of the antibacterial activity of vancomycin and 18β-glycyrrhetinic acid", Journal of Drug Delivery Science and Technology, 2021 Publication	<1%
6	Mahir Mohammed, Usri H. Ibrahim, Aimen Aljoundi, Calvin A Omolo et al. "Enzyme-responsive biomimetic solid lipid nanoparticles for antibiotic delivery against hyaluronidase-secreting bacteria", International Journal of Pharmaceutics, 2023 Publication	<1%
7	Mohammed Salih, Calvin A. Omolo, Nikita Devnarain, Ahmed A. Elrashedy et al. " Supramolecular self-assembled drug delivery system (SADDs) of vancomycin and	<1%

tocopherol succinate as an antibacterial agent: , and evaluations ", Pharmaceutical Development and Technology, 2020
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11 Calvin A. Omolo, Rahul S. Kalhapure, Nikhil Agrawal, Sanjeev Rambharose, Chunderika Mocktar, Thirumala Govender. "Formulation <1%

and Molecular Dynamics Simulations of a Fusidic Acid Nanosuspension for Simultaneously Enhancing Solubility and Antibacterial Activity", Molecular Pharmaceutics, 2018
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12 Ragi Jadimurthy, Shilpa Borehalli Mayegowda, S.Chandra Nayak, Chakrabhavi Dhananjaya Mohan, Kanchugarakoppal S. Rangappa. "Escaping mechanisms of ESKAPE pathogens from antibiotics and their targeting by natural compounds", Biotechnology Reports, 2022 <1%
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14 Ahmed Youssef, Narendar Dudhipala, Soumyajit Majumdar. "Ciprofloxacin Loaded Nanostructured Lipid Carriers Incorporated into In-Situ Gels to Improve Management of Bacterial Endophthalmitis", Pharmaceutics, 2020 <1%
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15 Sergey Zaichik, Christian Steinbring, Claudia Menzel, Ludwig Knabl et al. "Development of self-emulsifying drug delivery systems <1%

(SEDDS) for ciprofloxacin with improved mucus permeating properties", International Journal of Pharmaceutics, 2018
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16	"Efflux-Mediated Antimicrobial Resistance in Bacteria", Springer Science and Business Media LLC, 2016 Publication	<1%
17	Kaio Jefté Santos De Oliveira Dias, Gustavo Marinho Miranda, Jonatas Reis Bessa, Ana Carolina Justino De Araújo et al. "Terpenes as bacterial efflux pump inhibitors: A systematic review", Frontiers in Pharmacology, 2022 Publication	<1%
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23	Maria Anndressa Alves Agreles, Iago Dillion Lima Cavalcanti, Isabella Macário Ferro Cavalcanti. "The Role of Essential Oils in the Inhibition of Efflux Pumps and Reversion of Bacterial Resistance to Antimicrobials", Current Microbiology, 2021 Publication	<1%
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26	Samreen, Faizan Abul Qais, Iqbal Ahmad. " screening and validation of phytocompounds as multidrug efflux pump inhibitor against ",	<1%

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27	Alanood S Almurshedi, Hessah A Aljunaidel, Bushra Alquadeib, Basmah N Aldosari et al. "Development of Inhalable Nanostructured Lipid Carriers for Ciprofloxacin for Noncystic Fibrosis Bronchiectasis Treatment", International Journal of Nanomedicine, 2021 Publication	<1%
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31	C.I. Mawang, Y.Y. Lim, K.S. Ong, A. Muhamad, S.M. Lee. " Identification of α-tocopherol as a bioactive component of with disrupting property against preformed biofilm of ", Journal of Applied Microbiology, 2017 Publication	<1%

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36	Gulrukh Iqbal, Sulaiman Faisal, Sara Khan, Dilawar Farhan Shams, Akhtar Nadhman. "Photo-inactivation and efflux pump inhibition of methicillin resistant Staphylococcus aureus using thiolated cobalt doped ZnO nanoparticles", Journal of Photochemistry and Photobiology B: Biology, 2019 Publication	<1%
37	Sara Cunha, Ben Forbes, José Manuel Sousa Lobo, Ana Catarina Silva. "Improving Drug Delivery for Alzheimer's Disease Through Nose-to-Brain Delivery Using Nanoemulsions, Nanostructured Lipid Carriers (NLC) and in situ Hydrogels", International Journal of Nanomedicine, 2021 Publication	<1%
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39	New Weapons to Control Bacterial Growth, 2016. Publication	<1%
40	Noha Badawi, Khalid El-Say, Dalia Attia, Mohamed El-Nabarawi, Mohey Elmazar, Mahmoud Teaima. "Development of Pomegranate Extract-Loaded Solid Lipid Nanoparticles: Quality by Design Approach to Screen the Variables Affecting the Quality	<1%

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44 Arya Nair, Rashmi Mallya, Vasanti Suvarna, Tabassum Asif Khan, Munira Momin, Abdelwahab Omri. "Nanoparticles–Attractive Carriers of Antimicrobial Essential Oils", Antibiotics, 2022 <1%
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45 Nisar Sayyad, Ruma Maji, Calvin A. Omolo, Ab Majeed Ganai et al. "Development of niosomes for encapsulating captopril-quercetin prodrug to combat hypertension", International Journal of Pharmaceutics, 2021 <1%
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46	Ruma Maji, Calvin A. Omolo, Yajna Jaglal, Sanil Singh, Nikita Devnarain, Chunderika Mocktar, Thirumala Govender. "A transferosome-loaded bigel for enhanced transdermal delivery and antibacterial activity of vancomycin hydrochloride", International Journal of Pharmaceutics, 2021 Publication	<1%
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