DEVELOPMENT OF AN ANTIMICROBIAL PREPARATION USING MICROEMULSION-BASED SILVER NANOPARTICLES

NIRATCHA CHAISOMBOON

A Thesis Submitted to the Graduate School of Naresuan University

in Partial Fulfillment of the Requirements

for the Master of Science in Pharmacology and Biomolecular Sciences

2024

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by Niratcha Chaisomboon

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Pharmacology and Biomolecular Sciences of Naresuan University

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Academic Paper M.S. Thesis in Pharmacology and Biomolecular Sciences, Naresuan University, 2024
Keywords Silver nanoparticles, microemulsion, antimicrobial resistance

ABSTRACT

Background

The increasing prevalence of antimicrobial resistance necessitates the development of alternative therapeutic strategies. Silver nanoparticles (AgNPs) have demonstrated broad-spectrum antimicrobial activity, yet formulation challenges limit their clinical translation. This study aims to develop and evaluate an AgNP microemulsion as a novel localized antimicrobial therapy, focusing on its formulation stability, antimicrobial efficacy, and biocompatibility.

Methods

A silver nanoparticle microemulsion was formulated using a bottom-up synthesis approach, stabilized with polyvinyl alcohol (PVA) and Tween 80, and characterized for particle size, zeta potential, and UV-visible spectroscopy. Antimicrobial efficacy was assessed against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus mutans* using disk diffusion and broth dilution assays. Cytotoxicity was

evaluated in L929 fibroblast cells using the MTT assay to establish a therapeutic window.

Results

The AgNP microemulsion exhibited a mean particle size of 175.63 ± 0.31 nm, with a zeta potential of -1.03 ± 0.04 mV, indicating moderate colloidal stability. UV-visible spectroscopy confirmed nanoparticle formation, with a plasmon resonance peak at 230 nm. Antimicrobial testing revealed limited efficacy, with inhibition zones of 9.50 mm (*S. aureus*), 9.53 mm (*P. aeruginosa*), and 11.89 mm (*S. mutans*), significantly lower than 0.2% chlorhexidine. MIC and MBC values exceeded 0.7 mg/mL, suggesting suboptimal bactericidal potency. Cytotoxicity studies demonstrated >70% cell viability at concentrations ≤16 µg/mL, but significant toxicity at 32 µg/mL, indicating a narrow therapeutic window.

Conclusion

This study highlights the potential of AgNP microemulsions as a localized antimicrobial alternative but emphasizes the need for formulation optimization to enhance bactericidal efficacy while minimizing cytotoxicity. Future studies should explore surface modifications, synergistic agents, and controlled-release strategies to improve clinical applicability.

ACKNOWLEDGEMENT

This thesis would not have been possible without the gracious support and kind assistance of many individuals, to whom I am deeply gateful.

First and foremost, I would like to express my sincere and profound gratitude to Associate Professor Dr. Prayuth Poowaruttanawiwit, my principal advisor, for his invaluable guidance, insightful suggestions, and meticulous attention throughout this research. His dedication, patience, and encouragement have been instrumental in shaping the academic quality of this work. I am truly indebted to his unwavering support and would like to extend my heartfelt appreciation.

I am equally grateful to Associate Professor Dr. Nattakanwadee Khumpirapang, my first co-advisor, for her expert advice and constructive feedback in the fields of pharmaceutical chemistry and pharmacognosy, which significantly enhanced the quality and depth of this study.

My sincere thanks also go to Assistant Professor Dr. Chanida Chantim, my second co-advisor, for her generous guidance and perspectives in the field of otorhinolaryngology. Her expertise has contributed greatly to broadening the scope and relevance of this research.

I wish to thank Dr. Hathairat Lekatan, my third co-advisor, for her invaluable support and advice in cell and tissue culture techniques. Her continuous encouragement and guidance provided me with a truly enriching learning experience throughout this journey.

The collective knowledge and experience of my co-advisors, each with distinct expertise, provided a rare and invaluable foundation for this research. Their complementary insights have inspired and shaped my development both academically and personally.

ACKNOWLEDGEMENT (CONT.)

I am sincerely thankful to Associate Professor Dr. Acharaporn Duangjai, the chairperson of my thesis examination committee, for her time, constructive criticisms, and valuable suggestions, which greatly contributed to the final refinement of this thesis.

I am deeply grateful to Assistant Professor Dr. Damrongsak Pekthong, who graciously served as a committee member for my thesis defense and provided invaluable guidance and suggestions that significantly enhanced the quality and completeness of this thesis.

I would like to acknowledge the Faculty of Pharmaceutical Sciences, Naresuan University, for granting me a master's degree scholarship, enabling me to devote myself fully to my academic and research pursuits.

My appreciation also extends to the Graduate School of Naresuan University, for awarding the 2025 High-Potential Graduate Research Grant (Round 1), which supported the execution of this research project.

I wish to thank all the professors and staff at the Faculty of Pharmaceutical Sciences, Naresuan University, for imparting their knowledge and offering their support during my studies.

I am truly grateful to the Faculty of Dentistry, Naresuan University, my current affiliation, for granting me permission to pursue graduate studies during official working hours, thereby facilitating my academic and professional growth.

Special thanks are due to the Research Laboratory, Research and Innovation Division, Faculty of Dentistry, for kindly allowing me access to facilities and equipment throughout my research. I am also thankful to the laboratory staff for their generous assistance and insightful guidance.

ACKNOWLEDGEMENT (CONT.)

I am deeply grateful to Associate Professor Dr. Ruchadaporn Kaomongkolgit for kindly providing the *Candida albicans* ATCC 10231 strain, which played a vital role in the completion of this study.

My heartfelt appreciation goes to my colleagues, whose encouragement and support enriched both my work and academic journey with meaning and fulfillment. To my fellow graduate students, especially Ms. Nawarad Gimiphan, thank you for your friendship, encouragement, collaboration, and for sharing thoughts and experiences that made this journey more memorable.

Finally, I extend my deepest gratitude to my beloved parents, for their unconditional love, invaluable teachings, and unwavering support throughout my life. I also thank my brother for always standing by me. This accomplishment is truly the result of the love and encouragement I have received from my cherished family.

May the benefits derived from this thesis serve as a humble tribute to my parents, my teachers, and all benefactors who have laid the foundation of my education and inspired me along the way.

Niratcha Chaisomboon

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CHAPTER I

INTRODUCTION

Rationale and Problem Statement

The global rise of antimicrobial resistance (AMR) represents an urgent and growing threat to public health, prompting the need for novel antimicrobial therapies that are not only effective and safe, but also less susceptible to resistance development (1,2). Although conventional antibiotics remain widely used, their repeated and often indiscriminate application exerts selective pressure on microbial populations, accelerating the emergence of multidrug-resistant (MDR) strains (3,4). In addition, systemic antibiotic therapy is frequently associated with adverse effects, including hepatotoxicity, nephrotoxicity, systemic toxicity, and drug-drug interactions, thereby limiting their long-term clinical utility (5).

In response to these challenges, there is increasing interest in localized antimicrobial strategies that deliver therapeutic agents directly to the site of infection. Such approaches aim to achieve high local concentrations of antimicrobials, reduce systemic exposure, and consequently minimize toxicity.

Among the promising candidates for localized therapy are silver nanoparticles (AgNPs), which have attracted significant attention due to their broad-spectrum antimicrobial activity, multi-modal mechanisms of action, and low propensity for resistance development (6,7). Unlike traditional antibiotics, which typically target a single bacterial process, AgNPs act through multiple pathways—disrupting microbial membranes, inducing reactive oxygen species (ROS), and interfering with intracellular functions—thus making resistance development significantly less likely (8). Furthermore, AgNPs bypass hepatic metabolism and renal excretion, reducing the risk of systemic accumulation and toxicity (9). These pharmacokinetic advantages position AgNPs as ideal candidates for topical or localized applications, including wound care, oral infections, and ophthalmic conditions.

Despite their therapeutic potential, clinical translation of AgNPs is hindered by several formulation challenges, such as instability, agglomeration, and inconsistent delivery at the target site (10,11). A viable solution to these issues lies in the incorporation of AgNPs into microemulsion-based delivery systems, which are known

to improve nanoparticle dispersion, enhance stability, and promote retention at the site of infection. Microemulsions are thermodynamically stable colloidal systems that facilitate controlled release and increased interaction with microbial membranes, offering a strategic advantage in prolonging antimicrobial activity (12,13).

While AgNPs have demonstrated potent antimicrobial effects in various in vitro and preclinical models, their performance within a microemulsion matrix remains inadequately characterized (14–16). Furthermore, comparative studies against standard antiseptics such as chlorhexidine which, despite its effectiveness, carries notable cytotoxicity risks are necessary to determine the clinical relevance of AgNPs-based alternatives (17,18). Additionally, although AgNPs are generally considered biocompatible at controlled doses, the cytotoxic potential of AgNPs microemulsions on mammalian cells warrants rigorous investigation to ensure safety and therapeutic viability.

In summary, the integration of AgNPs into microemulsion delivery systems offers a promising pathway toward the development of a stable, effective, and safe localized antimicrobial therapy. However, comprehensive evaluation of its antimicrobial efficacy, biocompatibility, and comparative advantage over existing treatments remains essential to justify its clinical translation (18).

Research Question

Given these considerations, the central research question of this study is whether a microemulsion-based AgNPs formulation can serve as an effective and biocompatible alternative to conventional antimicrobial agents, addressing the need for localized antimicrobial therapy while minimizing systemic toxicity, drug resistance, and metabolic burden. To answer this question, the study focuses on three key objectives:

Objective of the Study

1. To develop and characterize of the microemulsion-based silver nanoparticles
2. To assess antimicrobial activity against clinically relevant bacterial pathogens of the microemulsions-based silver nanoparticles
3. To evaluate the cytotoxicity efficacy of the microemulsions-based silver nanoparticles

By systematically investigating the interplay between nanoparticle formulation, antimicrobial efficacy, and biocompatibility, this study aims to establish AgNPs

microemulsions as a next generation localized antimicrobial therapy, bridging the gap between preclinical innovation and clinical application in the fight against antibiotic resistance.

Hypothesis of the Study

Silver microemulsions, in which silver nanoparticles (AgNPs) or silver ions are dispersed within either the oil or aqueous phase of a microemulsion system, are hypothesized to possess broad-spectrum antimicrobial activity. The microemulsion platform not only enhances the stability and bioavailability of silver species but also enables their controlled and sustained release at the site of infection, thereby improving therapeutic efficacy. Given the intrinsic antimicrobial properties of silver effective against a wide range of bacteria, fungi, and viruses it is proposed that silver microemulsions will exhibit potent inhibitory activity across multiple microbial strains.

Furthermore, the study hypothesizes that while these formulations may demonstrate significant antimicrobial effects, their safety profile must be carefully evaluated. Specifically, cytotoxicity assays on relevant human cell lines will be employed to determine whether silver microemulsions elicit any adverse cellular responses, thus assessing their biocompatibility and clinical viability as localized antimicrobial agents (19).

Scope of the Study

This research focuses on the formulation, characterization, and biological evaluation of silver nanoparticle-based microemulsions for potential use as topical or localized antimicrobial agents. The scope includes:

- Development of stable microemulsion systems incorporating silver nanoparticles or silver ions
- Physicochemical characterization, including particle size, stability, and dispersity
- Evaluation of in vitro antimicrobial activity against selected bacterial and fungal pathogens
- Assessment of cytotoxic effects on relevant human cell lines to determine safety and tolerability

The study seeks to bridge the gap between antimicrobial efficacy and formulation safety, ultimately aiming to establish silver microemulsions as a viable alternative to conventional antimicrobial therapies.

CHAPTER II

LITERATURE REVIEW

Historical Context and Current Applications of Silver in Antimicrobial Use

Silver has a long and well-documented history of use as an antimicrobial agent. As early as antiquity, Herodotus described how Persian royalty stored drinking water in silver vessels to preserve its purity, especially during military campaigns where access to clean natural water was limited (20). In the medieval Islamic world, Avicenna (Ibn Sina) recorded the use of silver filings in 980 C.E. as a blood cleanser, specifically for treating symptoms such as heart palpitations and halitosis (21).

The antimicrobial potential of silver gained broader application during the 18th and 19th centuries, when colloidal silver was employed as a wound antiseptic and silver nitrate was widely used in burn management. Topically, silver nitrate was also administered to newborns to prevent gonococcal ophthalmia and was even ingested as a treatment for peptic ulcers through the early 20th century (20, 22).

Silver's antibacterial qualities were also acknowledged through its use in coins, tableware, and drinking vessels, a practice not only symbolic but functionally hygienic (23). In modern times, the element continues to play a role in hygiene and medicine. It is incorporated into textiles and personal sprays to inhibit odor-causing bacteria associated with perspiration (23-25) and used as a preservative or antimicrobial agent in cosmetic formulations (26). One of its most prominent roles in healthcare is in burn wound treatment, notably through silver sulfadiazine, a topical antimicrobial agent approved by the U.S. Food and Drug Administration (FDA) (27-29).

Ongoing research further supports silver's utility in clinical settings. It has been proposed for incorporation into medical and dental instruments, as well as implant coatings, to reduce infection risk (20, 30). Currently, over 300 clinical trials are underway or in recruitment phases, examining silver-containing compounds across a spectrum of medical applications (31). While most of these interventions remain confined to topical or surface-level use, they underscore silver's robust antibacterial profile and its potential for broader clinical deployment.

Silver Nanoparticles: Biological Properties and Emerging Applications

Nanoparticles are generally defined as particles with at least one dimension measuring less than 100 nanometers (nm) (32). These structures can vary widely in morphology, appearing as spheres, rods, cubes, tubes, and more complex geometries. Among the various types of bioactive nanoparticles, silver nanoparticles (AgNPs) have emerged as highly promising candidates due to their extensive antimicrobial efficacy (33).

AgNPs exhibit potent activity against a diverse array of bacteria, fungi, and viruses, and their ability to target multiple microbial pathways makes them less susceptible to resistance mechanisms (34). Recent years have seen a rapid increase in research exploring the applications of AgNPs, not only in infectious disease management but also in a range of other scientific disciplines. These include wound healing, retinal therapy, and even oncology, where AgNPs are under investigation for their anticancer and antioxidant properties (35).

Characterization of Silver Nanoparticles

Thorough characterization of silver nanoparticles is critical for understanding their behavior, stability, and interactions in biological and pharmaceutical systems. A variety of analytical techniques are utilized to determine their physical, chemical, and structural properties, including:

- UV–Visible spectroscopy
- Fourier Transform Infrared (FTIR) spectroscopy
- X-ray diffraction (XRD)
- X-ray photoelectron spectroscopy (XPS)
- Dynamic Light Scattering (DLS)
- Scanning Electron Microscopy (SEM)
- Energy-Dispersive X-ray Spectroscopy (EDX/EDS)
- Atomic Force Microscopy (AFM)
- Transmission Electron Microscopy (TEM)
- Particle Size Analyzer (PSA)
- Selected Area Electron Diffraction (SAED)
- Thermogravimetric Analysis (TGA)
- Nanoparticle Tracking Analysis (NTA) (36–38)

These methods assess various parameters such as particle size, morphology, zeta potential, crystallinity, surface area, porosity, solubility, and aggregation behavior. A commonly used preliminary indicator of AgNPs synthesis is the appearance of a brownish coloration upon the reduction of silver salts by plant extracts or other reducing agents. This color change is due to the surface plasmon resonance (SPR) phenomenon—an optical signature typically detected in the 400–500 nm range via UV–Vis spectrophotometry, caused by the collective oscillation of conduction band electrons in resonance with incident light (39, 40).

Table 1 Common techniques for the characterization of nanoparticles.

Techniques	Characterization techniques	Information provided	References
Spectroscopic techniques	UV– visible	Optical properties, synthesis, and stability of NPs	(39, 40)
	FTIR	Investigate phytochemical's role in NPs synthesis	(41, 42)
	DLS	Determine hydrodynamic diameter, polydispersity index of NPs	(42, 43)
X ray-based techniques	XRD, XAS, XRF, XPS	Determine crystalline structure and particle size of NPs	(44)
	AFM	Surface morphology, shape, size, electrical, and mechanical properties of NPs	(45)
Microscopic	SEM	Particle size distribution, morphology and topography of NPs	(46, 47)
	TEM	Morphology, shape, size, elemental composition and electrical conductivity NPs	(48)

Source: Results in Chemistry, 2023

Silver Nanoparticles and Antibacterial Activity

Antibiotics have historically served as the cornerstone in the treatment of bacterial infections. However, the accelerating emergence of multidrug-resistant (MDR) bacterial strains has created a pressing global health concern, rendering conventional antibiotics increasingly ineffective against many infections. Compounding this issue is the lengthy and costly process of developing new antibiotic agents. In response, the field of nanotechnology and specifically the use of silver nanoparticles (AgNPs) has garnered considerable attention as a viable alternative for addressing the threat of antimicrobial resistance (49).

Mechanisms of Antibacterial Action of Silver Nanoparticles

Silver nanoparticles exert broad-spectrum antimicrobial activity, effectively targeting both Gram-positive and Gram-negative bacteria, including strains resistant to multiple drugs. Although the exact mechanisms underlying their bactericidal effects are not yet fully elucidated, several interrelated pathways have been proposed to explain their efficacy (50) (see Figure 1).

According to Jones and Hoek (51), one key mechanism involves the release of free silver ions (Ag^+) from the nanoparticle surface. These ions are taken up by bacterial cells, where they can interfere with essential biological functions—disrupting ATP synthesis, damaging DNA replication machinery, and inhibiting other vital enzymatic activities. Simultaneously, AgNPs promote the generation of reactive oxygen species (ROS), which induce oxidative stress and contribute to cellular damage.

Another well-documented effect is direct membrane disruption. Silver nanoparticles can attach to and penetrate bacterial cell walls, particularly in Gram-negative organisms, where they induce pore formation and membrane destabilization. This results in increased membrane permeability, leakage of intracellular contents, and ultimately cell death. Additionally, silver ions contribute to the denaturation and oxidation of membrane proteins, damage to internal organelles, and eventual cell lysis.

Moreover, AgNPs have been shown to interfere with bacterial signal transduction pathways. Specifically, they alter the phosphotyrosine phosphorylation profile of

intracellular peptides, thereby impairing key regulatory signals necessary for bacterial growth and proliferation (50, 52).

Through this combination of chemical, physical, and biochemical mechanisms, silver nanoparticles deliver a multifaceted assault on microbial cells. This multi-targeted mode of action not only enhances their efficacy but also reduces the likelihood of resistance development making AgNPs highly attractive candidates in the search for next-generation antimicrobial agents.

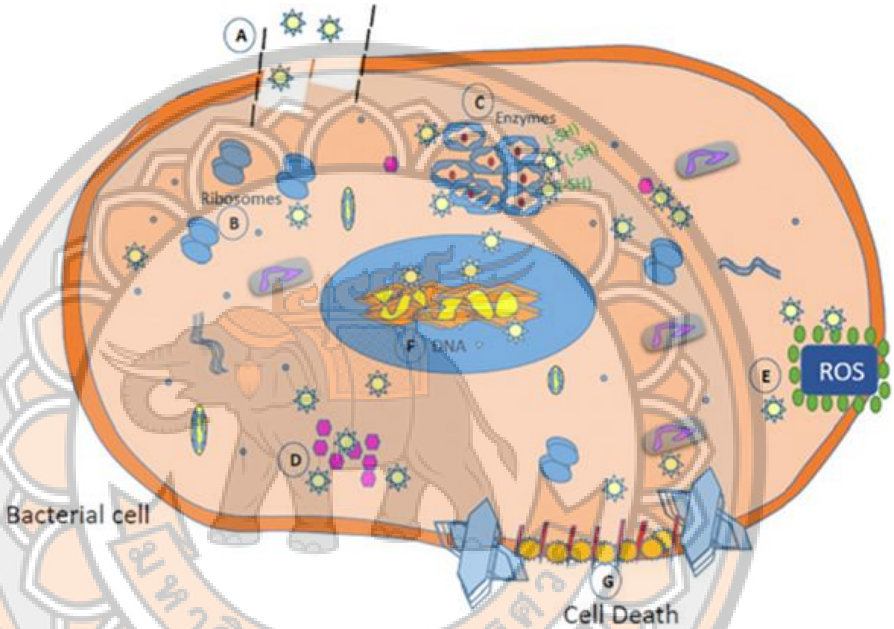

Figure 1 The antimicrobial action of silver nanoparticles

Source: Materials Science and Engineering: C, 2018

Figure 1 outlines the sequential events involved in the antibacterial activity of silver nanoparticles (AgNPs) through a multi-targeted mechanism of action:

- (A) Silver nanoparticles first diffuse through the bacterial envelope and accumulate at the plasma membrane, where their dissolution leads to localized release of Ag^{+} ions, compromising membrane integrity and causing leakage of cytoplasmic contents.
- (B) Internally, AgNPs interfere with ribosomal function by inducing ribosomal destabilization and denaturation, thereby inhibiting protein synthesis and exacerbating membrane degradation.

- (C) Silver nanoparticles subsequently interact with critical respiratory enzymes, binding to thiol (-SH) groups, leading to enzyme inactivation and disruption of metabolic pathways.
- (D) AgNPs interfere with the electron transport chain, impairing energy production and altering cellular signaling pathways.
- (E) Within the cell, mitochondrial damage contributes to the production of reactive oxygen species (ROS), resulting in oxidative stress and protein oxidation.
- (F) Silver nanoparticles also bind to bacterial DNA, hindering replication and transcription, ultimately triggering apoptotic-like death.
- (G) The cumulative effects lead to the formation of membrane indentations and perforations, causing the release of cellular organelles and culminating in cell lysis and death.

Size-Dependent and Dose-Dependent Antibacterial Activity

The principal antimicrobial mechanism of silver nanoparticles is mediated by the release of Ag^+ ions, which exert potent bactericidal effects. Notably, smaller nanoparticles (<10 nm) demonstrate higher ion release rates and enhanced interaction with bacterial membranes compared to larger particles (53). The minimum inhibitory concentration (MIC) of AgNPs varies across bacterial species:

- *Fusobacterium nucleatum*: ~0.003 mg/mL
- *Streptococcus mutans*: ~0.04 mg/mL (54)
- *Actinomyces oris*: ~0.5 mg/mL (55)

In a study by Sondi et al. (2004), complete inhibition of *Escherichia coli* growth was observed at AgNP concentrations of 50–60 μg/cm³, underscoring their potent antimicrobial capacity (56). Furthermore, nanoparticles in the 1-10 nm size range demonstrate direct interaction with bacterial cell membranes, significantly disrupting membrane permeability and structural integrity (57). These findings reinforce the critical influence of particle size and concentration on the bactericidal efficacy of silver nanoparticles.

Antifungal Activity of Silver Nanoparticles

Silver nanoparticles (AgNPs) have demonstrated broad-spectrum antifungal properties, with reported efficacy against 44 distinct fungal strains across multiple species (58). In particular, their antifungal action against *Candida albicans* a common opportunistic pathogen is believed to involve the disruption of cell membrane integrity, thereby impeding fungal growth and viability.

One of the primary antifungal mechanisms of AgNPs is associated with structural alterations at the biofilm level, where AgNPs interfere with biofilm architecture and formation, a critical factor in fungal pathogenicity and drug resistance (59). Several studies have highlighted the role of reactive oxygen species (ROS) in this process. The accumulation of ROS within fungal cells is known to induce oxidative stress, ultimately leading to apoptosis-like cell death.

Beyond membrane damage and ROS generation, Babele et al. demonstrated that AgNPs exert profound effects on fungal cell physiology, including alterations in the transcriptome, epigenome, and metabolome, thereby impairing essential biological processes (60). Vazquez-Muñoz et al. further proposed that the reduction of Ag^{+} ions outside the fungal cell plays a pivotal role in initiating cell death mechanisms, likely through intracellular stress pathways (61).

In a related study, Rozhin et al. provided genomic evidence suggesting that AgNPs interfere with the expression of genes involved in the maintenance of cell wall and membrane integrity, vesicular transport, oxidative metabolism, cellular respiration, and copper homeostasis all of which are vital for fungal survival (62, 63).

These findings are summarized schematically in Figure 2, which illustrates the complex and multifaceted antifungal actions of silver nanoparticles, ranging from cell membrane disruption and biofilm destabilization to genomic and metabolic dysregulation.

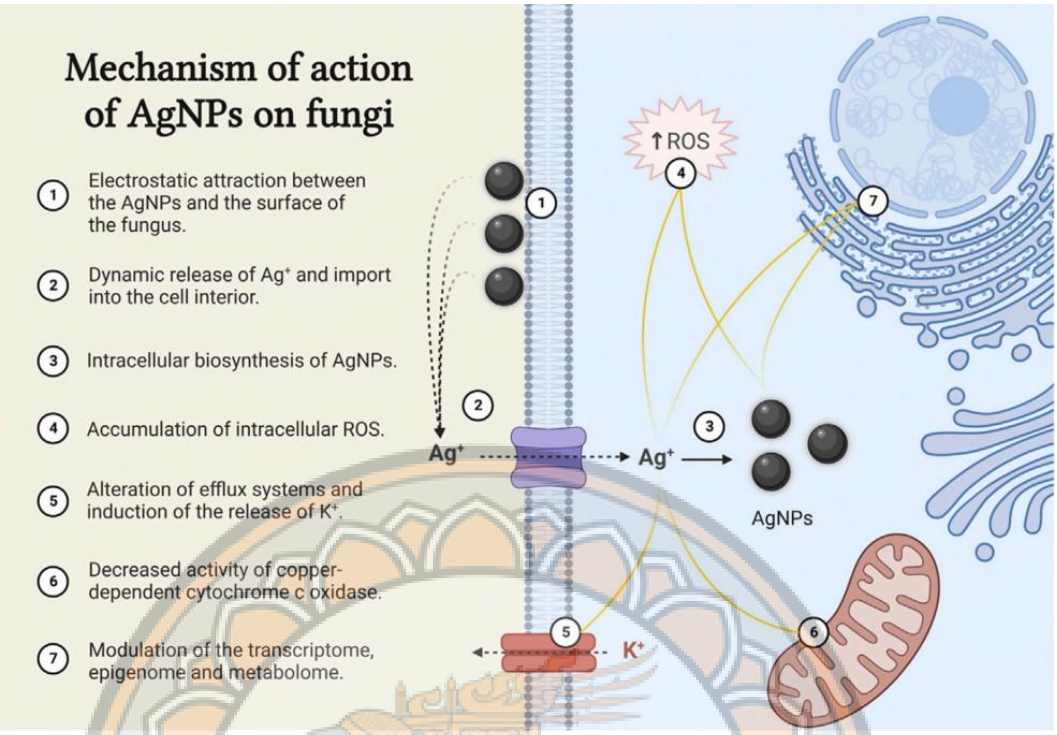

Figure 2 Antifungal mechanism of AgNPs. Reproduced with permission from Ref. (Copyright 2022 Frontiers)

Source: Frontiers in Chemistry, 2022

Factors Influencing the Antimicrobial Efficacy of Silver Nanoparticles

The antimicrobial performance of silver nanoparticles (AgNPs) is modulated by several physicochemical and environmental parameters, notably the type of metal precursor, nanoparticle size, and conditions during synthesis. Among these, particle size plays a pivotal role. AgNPs with diameters less than 10 nm, particularly those approaching 5 nm, demonstrate enhanced antibacterial activity due to their increased surface area-to-volume ratio, which facilitates greater interaction with microbial membranes (1).

Chemical synthesis techniques offer superior control over nanoparticle size and morphology, enabling precise tuning to maximize antimicrobial properties (2). Although green synthesis and physical methods may yield particles with slightly more heterogeneity in size, effective antimicrobial action remains achievable if the particle size remains within the sub-10 nm range (3). Independent of synthesis route, AgNPs near 5 nm in size consistently exhibit optimal antimicrobial performance (4).

Environmental parameters also significantly influence nanoparticle formation and function. For instance, temperature affects nanoparticle morphology: higher temperatures tend to produce spherical particles, while lower temperatures favor anisotropic forms such as nanotriangles. For biogenic synthesis, the optimal temperature window ranges between 25°C and 37°C (5). The pH of the reaction medium also dictates stability, with basic conditions favoring colloidal stability. However, extreme alkalinity (pH >11) may lead to particle aggregation and diminished efficacy (6). Moreover, reaction time impacts the extent of metal ion reduction, where shorter durations can result in a higher concentration of smaller nanoparticles (7). Together, these factors determine not only the size and shape of the nanoparticles but also their biological activity, with smaller, well-dispersed AgNPs demonstrating superior antimicrobial performance.

Broad-Spectrum Antimicrobial Activity of Silver Nanoparticles

Silver nanoparticles possess well-documented broad-spectrum antimicrobial activity, exhibiting potent effects against a variety of bacteria, fungi, and viruses. In bacterial systems, AgNPs have shown efficacy against both Gram-positive and Gram-negative strains, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, and *Salmonella typhi*. Their antifungal capabilities extend to species such as *Candida albicans* and *Aspergillus niger*, while antiviral activity has been observed against enveloped viruses like the Tacaribe virus, where AgNPs significantly suppressed viral RNA replication (64, 65).

The mechanism of action differs by microorganism. In bacteria, AgNPs disrupt the cell wall and membrane, leading to cell lysis. They also interfere with protein synthesis and DNA replication, primarily through the generation of reactive oxygen species (ROS) that cause oxidative damage. In fungi, AgNPs compromise membrane integrity and inhibit budding and morphogenesis, crucial steps in fungal proliferation. For viruses, AgNPs interact with the viral envelope or surface proteins, thereby blocking viral entry into host cells and impeding replication. These antimicrobial actions are summarized in Table 2. (64, 65)

Minimum Inhibitory and Bactericidal Concentrations of Silver Nanoparticles

The antimicrobial potency of AgNPs is frequently quantified using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. Chemically synthesized AgNPs have demonstrated MIC values ranging from 0.84 to 6.75 µg/mL against key bacterial pathogens including *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, with corresponding MBC values generally exceeding the MIC (66). These results indicate that AgNPs are capable of both inhibiting bacterial proliferation and inducing cell death, with efficacy influenced by synthesis method, particle characteristics, and microbial strain.

Table 2 Antimicrobial Activity and Mechanisms of Action of Silver Nanoparticles (AgNPs)

Microorganism Type	Microorganisms	Mechanism of Action
Bacteria	*E. coli, P. aeruginosa, S. aureus, B. subtilis, K. pneumoniae, S. typhi*	Disruption of cell membrane and wall, inhibition of protein synthesis and DNA replication, ROS generation.
Fungi	*C. albicans, A. niger*	Disturbance of cell membrane integrity, inhibition of budding process.
Viruses	Tacaribe virus	Attachment to virus surface, prevention of entry into host cells, reduction in viral RNA production.

Toxicity of Silver Nanoparticles

Silver ions have long demonstrated antimicrobial efficacy, and this property has been further enhanced by the advent of silver nanoparticle (AgNPs) formulations, which offer a significantly increased surface area, thereby improving interaction with microbial targets. This nanoparticle-enabled enhancement has shown effectiveness not only against conventional pathogens but also antibiotic-resistant strains, and even

produces synergistic effects when used alongside traditional antimicrobial agents (67–71).

However, the same properties that make AgNPs potent antimicrobials may also contribute to cellular toxicity under certain conditions. In vitro studies using rat hepatocytes have reported oxidative stress and impaired mitochondrial function upon exposure to AgNPs, leading to hepatic tissue damage. In vivo rodent studies have identified a No Observable Adverse Effect Level (NOAEL) of 30 mg/kg and a Lowest Observable Adverse Effect Level (LOAEL) of 125 mg/kg, establishing important reference points for safety evaluation (72).

Mechanistically, the generation of reactive oxygen species (ROS) through disruption of the mitochondrial respiratory chain is central to AgNPs-induced cytotoxicity. This oxidative insult impairs ATP synthesis, induces apoptotic signaling, and causes DNA fragmentation, all of which compromise cell viability (73). Interestingly, prolonged nasal exposure to silver nanoparticles over a 90-day period did not elicit genotoxic effects in animal models, regardless of sex, suggesting route-specific toxicity profiles (68).

Further research has linked AgNP exposure to membrane permeability changes, resulting in electrophysiological disruptions such as altered action potentials in excitable tissues (74). Moreover, immunotoxic and apoptotic pathways, involving modulation of caspase activity and mitochondrial stress signaling (e.g., JNK pathway), have also been implicated in AgNPs-induced cellular damage (75). Although these findings raise important concerns, additional controlled in vivo studies at defined dosage thresholds are necessary to corroborate and contextualize these in vitro observations.

Microemulsions as a Drug Delivery Platform

Microemulsions are lipid-based, thermodynamically stable colloidal systems that have shown considerable promise in enhancing topical and transdermal drug delivery. Though structurally similar to nanoemulsions, microemulsions differ in key aspects such as droplet size (100–400 nm) and their inherent thermodynamic stability. These systems typically consist of an oil phase dispersed in water (oil-in-water, o/w),

stabilized by surfactants and co-surfactants, allowing for efficient encapsulation and solubilization of lipophilic therapeutic agents.

Topical delivery via microemulsions offers multiple advantages over systemic routes, particularly for poorly water-soluble compounds. By bypassing first-pass hepatic metabolism, these systems enhance bioavailability and reduce systemic side effects. In dermatological applications, site-specific drug targeting is of particular value, as it enables localized therapy with lower drug concentrations, minimizing both toxicity and drug resistance. Owing to their versatility and ability to penetrate lipophilic barriers, microemulsions have been widely adopted for the delivery of synthetic and natural compounds, improving stability, pharmacokinetics, and bioactivity (76).

Bridging Innovation and Application: The Promise of AgNP Microemulsions

Given the dual need to enhance antimicrobial efficacy while mitigating systemic toxicity, silver nanoparticle-based microemulsions offer a compelling strategy for next-generation localized antimicrobial therapy. By systematically investigating the interplay between nanoparticle formulation, antimicrobial potency, and biocompatibility, this study aims to establish AgNPs-loaded microemulsions as a clinically viable alternative in the fight against antimicrobial resistance. This approach bridges the translational gap between preclinical innovation and therapeutic application, offering a platform that is effective, targeted, and safe for localized infections where systemic treatments fall short.

CHAPTER III

RESEARCH METHODOLOGY

Ethical Approval

The study protocol was reviewed and approved by the Biosafety Committee of Naresuan University, Phitsanulok, Thailand. The research adhered to all institutional, national, and international guidelines for the safe handling of nanoparticle-based materials. Approval was granted under the reference number NUIBC MI 67-11-65, ensuring compliance with biosafety and ethical standards throughout all phases of the study, including formulation, antimicrobial efficacy testing, and cytotoxicity assessments. This approval certifies that all procedures were conducted in a manner that ensures the safety of researchers, test systems, and the surrounding environment.

1. Research Instruments and Materials

1.1 Research Instruments

The following instruments were employed for formulation, characterization, and biological testing:

- Zetasizer (Malvern, UK)
- UV-Visible Spectrophotometer (Thermo Scientific®, USA)
- Biological Safety Cabinet, Class II Type A2 (LABCONCO, USA)
- Microplate Absorbance Spectrophotometer (xMark™, Bio-Rad®, USA)
- Inverted Microscope (IX70, Olympus®, Japan)
- Digital Camera System (DP72, Olympus®, Japan)
- CO_2 Incubator (Thermo Scientific®, USA)
- General Incubator (Thermo Scientific®, USA)
- Microcentrifuge (Hettich, Germany)
- Water Bath (J.P. SELECTA, Spain)
- Autoclave (STURDY, Taiwan)
- Hot Air Oven (Memmert, Germany)
- Analytical Balance (Mettler-Toledo, Switzerland)
- pH Meter (Mettler-Toledo, Switzerland)
- Magnetic Hotplate Stirrer (IKA, Germany)
- Micropipettes (Gilson®, USA)

- 8-Channel Multichannel Pipette (Eppendorf, Germany)
- Mobile Pipetting Aid (Thermo Scientific®, USA)
- Vortex Mixer (Labnet, USA)
- Culture Plates (Nunc, Denmark)
- 96-Well Microplates
- Sterile Pipette Tips
- Plastic Sterile Inoculating Loops
- Semi-Micro Cuvettes (1.6 mL)
- Beaker Glassware
- Laboratory Bottles
- Flasks
- Centrifuge Tubes (0.5 mL, 15 mL, and 50 mL)

1.2 Research Materials and Chemical Agents

- Mueller Hinton Agar (MHA), Brain Heart Infusion (BHI), Sabouraud Dextrose Agar (SDA) (Difco, BBL™, USA)
- Phosphate Buffered Saline (PBS)
- Dulbecco's Modified Eagle Medium (DMEM and DMEM without phenol red) (Hyclone, USA)
- 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin, 1% L-glutamine (Hyclone, USA)
- 0.25% Trypsin-EDTA (Hyclone, USA)
- MTT reagent (Sigma®, USA)
- Dimethyl Sulfoxide (DMSO) (RCI Labscan, Ireland)
- Silver Nanoparticles (Chanjao Longevity Co., Ltd., Thailand)
- Polyvinyl Alcohol (PVA, commercial grade)
- Tween 80 (RCI Labscan, Ireland)
- Ethanol (Merck, Germany)

2. Research Methodology

2.1 Formulation and Characterization of Silver Nanoparticle Microemulsion

The silver nanoparticle microemulsion was prepared using a modified bottom-up approach, integrating polyvinyl alcohol (PVA) and Tween 80 as stabilizing agents to enhance colloidal stability and minimize aggregation. The process employed

ultrasonication, ensuring homogeneous nanoparticle dispersion and improved bioavailability within the emulsion matrix.

Following formulation, comprehensive physicochemical characterization was conducted to assess stability, particle distribution, and suitability for biomedical application:

- Dynamic Light Scattering (DLS) analysis was performed to determine particle size and zeta potential, both critical for understanding nanoparticle–biological interactions.
- UV-Visible Spectrophotometry was used to monitor surface plasmon resonance (SPR), confirming nanoparticle synthesis and stability through characteristic absorption peaks.
- Stability studies were carried out under both accelerated and real-time conditions, evaluating potential changes in particle size, phase separation, and optical properties (e.g., colorimetric shifts) to ensure long-term formulation robustness.

Preparation of Silver Nanoparticle Microemulsion System

The silver nanoparticle solution, with a stock concentration of 7500 ppm (particle size: 40-90 nm; pH 6-8; water-soluble), was used for microemulsion preparation. The formulation followed a five-step protocol to ensure uniformity and reproducibility:

1. Base Solution Preparation:
   ~22 mL of ultrapure water (18.2 MΩ·cm at 25°C) was measured and combined with 0.05 g of polyethylene glycol (PEG) as a dispersing agent.
2. Nanoparticle Addition:
   ~0.025 g of citrate- or PVA-coated AgNPs was gradually added under continuous magnetic stirring to promote even distribution.
3. Stabilization and Surfactant Integration:
   0.125 g of PVA and 0.025 g of Tween 80 were incorporated, with continued stirring until fully mixed.
4. Volume Adjustment:
   Ultrapure water was added to reach a final volume of 25 mL.

5. Sterilization:

   The final solution was sterilized via filtration or autoclaving, ensuring microbial safety for downstream biological testing.

A schematic representation of this process is provided in Figure 3

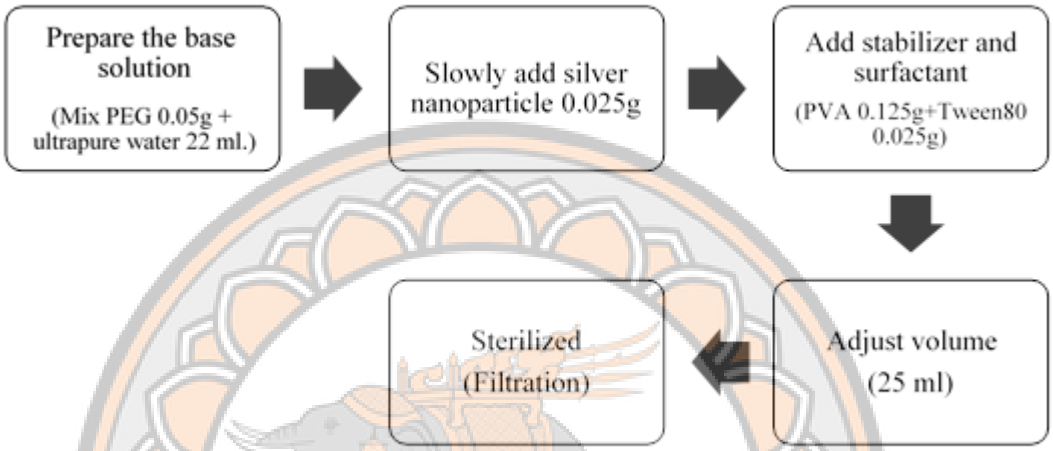

Figure 3 The preparation of the silver nanoparticle solution involved a five-step procedure

Characterization of Silver Nanoparticles within Microemulsion Systems

The characterization of silver nanoparticles (AgNPs) embedded in microemulsion systems was performed to evaluate their physicochemical stability, dispersibility, and overall suitability for biomedical applications. UV–visible spectrophotometry was conducted across a spectral range of 200-600 nm to investigate the optical properties of the nanoparticles. The presence of a characteristic surface plasmon resonance (SPR) peak confirmed the successful synthesis and stable incorporation of AgNPs within the microemulsion matrix.

In addition, dynamic light scattering (DLS) and zeta potential analysis were employed to determine particle size distribution and surface charge, respectively. These parameters are critical for assessing the colloidal stability and dispersion behavior of the nanoparticles in the microemulsion system. The zeta potential values, in particular, provided insight into the electrostatic repulsion between particles, which directly influences aggregation tendencies and long-term suspension stability.

Measurements were interpreted in conjunction with the refractive index of the system to ensure accuracy in sizing and charge estimations.

Together, these characterization methods offered a robust understanding of the structural and functional properties of AgNPs within the microemulsion platform, ensuring their reliability and reproducibility for downstream antimicrobial and cytotoxicity evaluations.

2.2 Antimicrobial Activity Testing

2.2.1 Preparation of Microbial Inoculums and Evaluation of the antibacterial activity

The antibacterial activity testing of silver nanoparticles within microemulsion systems was conducted using two primary methods: the disk diffusion method (77) and the broth dilution test.

1) Disk Diffusion Method

Antimicrobial Susceptibility Testing of Silver Microemulsions

The bacterial and fungal strains selected for this study were sourced from the American Type Culture Collection (ATCC) and included:

- *Staphylococcus aureus* ATCC 25923
- *Pseudomonas aeruginosa* ATCC 27853
- *Streptococcus mutans* A32-2
- *Candida albicans* ATCC 10231

To preserve viability, all microorganisms were routinely subcultured and maintained in appropriate growth media. Specifically, *S. aureus*, *P. aeruginosa*, and *S. mutans* were cultured on Brain Heart Infusion (BHI) agar, while *C. albicans* was grown on Sabouraud Dextrose Agar (SDA). Bacterial strains were incubated at 37°C for 24–48 hours under aerobic conditions, except for *S. mutans*, which required anaerobic incubation. *C. albicans* cultures were maintained at either 25°C for 48 hours or 37°C for 24 hours, depending on the experimental conditions.

Microbial inocula were prepared using the direct colony suspension method. For bacteria, suspensions were adjusted to an optical density (OD) of 0.08-0.10 at 625 nm, corresponding to a final concentration of approximately 1×10^8 CFU/mL (78). For

fungal suspensions, an OD of 0.70-0.90 at 530 nm was used to achieve a final inoculum density of 2×10^8 PFU/mL.

The antimicrobial activity of the silver nanoparticle microemulsion was evaluated using the disk diffusion method, following Clinical and Laboratory Standards Institute (CLSI) guidelines (79, 80). A 2-fold serial dilution of the microemulsion was prepared, ranging from 0.05 mg/mL to 0.7 mg/mL.

Mueller-Hinton Agar plates were inoculated with bacterial suspensions (1×10^8 CFU/mL) using sterile cotton swabs to evenly spread the cultures across the agar surface. Sterile 6 mm paper disks were individually loaded with 10 µL of each test concentration, allowed to air-dry, and then gently placed onto the inoculated agar plates. The disks were left undisturbed for 5 minutes to allow diffusion prior to incubation. Plates were incubated at appropriate conditions for 24-48 hours, depending on the organism.

Distilled water was used as a negative control, while 0.2% chlorhexidine served as the positive control for both bacterial and fungal strains. Following incubation, zones of inhibition surrounding each disk were measured using Vernier calipers, and antimicrobial efficacy was assessed based on the diameter of these zones, in accordance with CLSI interpretative criteria.

2) Broth Dilution Test

Determination of MIC and MBC Using the Microbroth Dilution Method

To quantitatively assess the antimicrobial efficacy of silver microemulsions, the microbroth dilution technique was employed in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (77). This method enables the determination of both the Minimum Inhibitory Concentration (MIC) defined as the lowest concentration of antimicrobial agent that visibly inhibits microbial growth and the Minimum Bactericidal Concentration (MBC), which represents the lowest concentration required to eliminate the microorganism.

The test samples were subjected to 2-fold serial dilutions, generating a concentration gradient across six wells of a sterile 96-well microtiter plate, with each well containing 100 µL of the diluted test agent. Subsequently, 100 µL of microbial suspension (adjusted to a final cell density of 1×10^8 CFU/mL) was added to each well, resulting in a final reaction volume of 200 µL per well. The assay included

broth-only wells as negative controls and broth with inoculum but no test agent as positive controls.

Following preparation, plates were incubated at optimal conditions specific to each microorganism (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, and *Candida albicans*) for 24-48 hours. MIC values were determined visually by assessing turbidity and optical density, with the MIC defined as the lowest concentration showing no visible microbial growth.

To determine the MBC, wells corresponding to concentrations with no visible growth were selected. A small aliquot (typically 10 µL) from each of these wells was transferred to appropriate agar plates using the drop plate method, and incubated under conditions specific to each organism. The MBC was recorded as the lowest concentration at which no colony formation was observed, indicating complete microbial killing (81).

While the disk diffusion assay provided initial insights into antimicrobial activity, it lacked the resolution required to definitively establish potency. Therefore, the microbroth dilution method was implemented to determine the MIC and MBC values of the silver microemulsion against *S. aureus*, *P. aeruginosa*, *S. mutans*, and *C. albicans*, offering a more precise and quantitative evaluation of its antimicrobial potential.

2.3 Cytotoxicity Evaluation in Mammalian Cells

2.3.1 Preparation of Cell Line:

L-929 Murine fibroblast cell line (CLS order no. 400260, Germany) cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained at 37°C in an environment with 95% humidity and 5% CO_2, with media changes performed every 2 days. Upon reaching 80% confluence, the cell culture medium was discarded, and the cells were rinsed twice with 2 mL of sterile PBS (pH 7.2-7.4). The cells were then subjected to trypsinization with 0.25% trypsin-EDTA solution for 5 minutes, and the trypsin activity was halted by adding an equal volume of complete medium. The detached cells were transferred to a centrifuge tube and centrifuged at 3000 rpm for 5 minutes.

Finally, the cells were counted or seeded for the experiment and continued to be incubated at 37°C with 5% CO_2. The cell culture was monitored daily using an inverted microscope.

2.3.2 Sample Preparation:

The L-929 cells will be seeded into cell culture plate. After 16-18 h, the serum free medium will be replaced and cultured for another 6-8 h. L-929 cells will be treated with 0, 0.5, 1, 2, 4, 8, 16, 32 µg/mL of silver nanoparticle (AgNPs) Commercial and 0, 0.5, 1, 2, 4, 8, 16, 32 µg/mL of silver microemulsions. The microemulsion (without AgNPs) concentrations of 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2% will be performed as vehicle controls for AgNPs microemulsions treatments, respectively.

Cytotoxicity Assays:

The cytotoxicity of silver nanoparticles within microemulsion systems was assessed using the MTT assay, which evaluates cell viability or cellular metabolic activity by measuring mitochondrial activity. Viable cells with normal mitochondrial activity reduce the yellow tetrazolium salt MTT (methyl-thiazolyl-tetrazolium; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals using mitochondrial succinate dehydrogenase. This activity is detected by a spectrophotometer at 570 nm, with a dark purple color indicating higher cell viability.

MTT Assay:

L929 cell lines were seeded at a density of 1×10^5 cells per well in a 96-well plate. After 24 hours of incubation, cells were treated with 100 µL of various concentrations of silver microemulsion samples. Untreated cells were used as control. Following another 24 hours of incubation, the supernatant fluids were removed, and 100 µL of MTT solution (0.5 mg/mL) dissolved in DMEM without phenol red was added to each well. After 1 hour of incubation at 37°C, the MTT solution was removed, and the formazan crystals were solubilized by adding 100 µL of pure DMSO to each well. The absorbance was measured at 570 nm using a Microplate Absorbance Spectrophotometer, with pure DMSO serving as the blank.

Result Interpretation:

Absorbance was measured at 570 nm using a microplate absorbance spectrophotometer. The percentage of cell viability was calculated using the following equation:

$$\%\ \text{Cell viability} = \frac{\text{Optical density}_{570}\ \text{of treated cell}}{\text{Optical density}_{570}\ \text{of untreated cell}} \times 100$$

This research phase aims to determine the dose ranges that are safe for mammalian cell lines. These identified safe dose ranges will then be utilized in subsequent efficacy investigations. The cytotoxicity MTT assay was employed for this purpose due to its empirically confirmed effectiveness in assessing the safety of nanoparticles in cell lines and its ease of execution, making it suitable for obtaining preliminary safety data.

2.4 Data Analysis

To ensure experimental reliability and reproducibility, all experiments were conducted in triplicate, minimizing variability and enhancing the robustness of the findings. A comprehensive statistical analysis was applied to interpret the data accurately. Descriptive statistics were utilized, with results expressed as Mean ± Standard Deviation (SD) to summarize the experimental outcomes and highlight variations within the dataset. For comparative analysis, a one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test was employed to determine statistically significant differences in antimicrobial activity across different treatment groups. This method ensures that intergroup variations are appropriately assessed while controlling multiple comparisons. A significance threshold (p-value < 0.05) was established for all statistical tests, ensuring that any observed differences are scientifically meaningful and not due to random variation. These statistical approaches provide a rigorous framework for evaluating the efficacy and safety of the AgNPs microemulsion formulation, supporting its potential therapeutic application.

CHAPTER IV

RESULTS AND DISCUSSION

1. Characterization of the Silver Nanoparticle Microemulsion

The silver nanoparticle (AgNPs) microemulsion was successfully developed and underwent comprehensive physicochemical characterization to determine its pharmaceutical suitability, stability, and functional performance.

Particle size, determined via dynamic light scattering (DLS), revealed a mean hydrodynamic diameter of 175.63 ± 0.31 nm, firmly within the nanometric scale. This is a critical range for topical and localized drug delivery, as nanoparticles under 200 nm are known to improve skin penetration through disrupted epithelial barriers, enhance passive diffusion across tissues, and increase retention at the site of action. Nanometric sizing also supports formulation uniformity and long-term stability by reducing gravitational settling.

Zeta potential was measured at -1.03 ± 0.04 mV, indicating a relatively low surface charge and weak electrostatic repulsion between particles. While sufficient to provide minimal dispersion stability, this value is well below the ±30 mV threshold considered indicative of strong colloidal stability. This suggests that although immediate aggregation may be delayed, the system is at risk of phase separation or particle coalescence during long-term storage. Therefore, incorporation of steric stabilizers such as nonionic surfactants or polymeric excipients (e.g., poloxamers or PEGylated emulsifiers) may be required to provide entropic barriers and maintain homogeneity over time.

The pH value, measured at 5.12 ± 0.01, lies within the physiologically acceptable range for topical and mucosal applications. This is particularly relevant for antimicrobial formulations, where acidic pH can synergize with silver ion activity while minimizing irritation to the skin or mucosal tissues. Ensuring pH compatibility is essential for patient tolerability, epithelial barrier integrity, and minimizing formulation-induced dermatitis.

UV–visible spectroscopy confirmed the presence of AgNPs via a distinct surface plasmon resonance (SPR) peak at 230 nm. This optical signature validates the

successful synthesis of nanosilver within the formulation. While classical SPR peaks of spherical AgNPs typically appear around 400–450 nm, shifts in SPR wavelength to lower values can occur in microemulsion systems due to the nanoparticle size, shape anisotropy, local refractive index, or capping agent effects. Thus, this deviation still supports AgNPs presence, particularly within complex surfactant-rich microenvironments.

Taken together, these findings indicate that the AgNPs microemulsion possesses key physicochemical attributes for use as a localized antimicrobial agent. However, the low zeta potential suggests that further formulation optimization is required to improve colloidal stability for commercial or clinical deployment. Detailed characterization data are presented in Table 3.

Table 3 Shows the particle size, PdI and zeta potential value of silver microemulsion samples measured by Zetasizer

Test sample	Mean Particle Size (nm) ± SD	PdI± SD	Zeta potential (mV) ± SD
Silver microemulsion	175.63 ± 0.31	0.577 ± 0.00	-1.03 ± 0.04

Note; SD; standard deviation, n=3

Table 3 presents the results of dynamic light scattering (DLS) and electrophoretic light scattering (ELS) analyses for the physicochemical characterization of the silver microemulsion formulation. The mean particle size was measured at 175.63 ± 0.31 nm, confirming nanoscale dispersion within the formulation a desirable attribute that supports improved dermal penetration, enhanced surface area for antimicrobial activity, and colloidal stability. The polydispersity index (PdI) was reported as 0.577 ± 0.00, which indicates a relatively broad size distribution among the nanoparticulate components. Although values below 0.3 are generally indicative of monodispersity, a PdI above 0.5 suggests heterogeneity that may affect long-term physical stability and uniformity. Optimization of emulsifier systems or homogenization parameters may be required to reduce particle size variability.

The zeta potential was determined to be -1.03 ± 0.04 mV, reflecting a low surface charge and limited electrostatic repulsion between dispersed particles. This value falls significantly below the ±30 mV threshold commonly associated with strong colloidal stability, suggesting a potential risk for particle aggregation or phase separation during extended storage. While the formulation may remain physically stable in the short term, incorporation of steric stabilizers or charge-modifying excipients may be necessary to enhance dispersion stability over time. All values are expressed as mean ± standard deviation (SD), with measurements performed in triplicate (n = 3).

Pharmaceutical and Physicochemical Considerations in the Development of a Silver Nanoparticle (AgNP) Microemulsion

The successful formulation of a silver nanoparticle (AgNP) microemulsion for topical or localized antimicrobial therapy requires meticulous control over several critical physicochemical parameters. These parameters particle size, surface charge (zeta potential), pH, and nanoparticle integrity directly influence the formulation's stability, biocompatibility, tissue penetration, and antimicrobial efficacy.

In the present formulation, the mean hydrodynamic diameter of the AgNPs was 175.63 ± 0.31 nm, as determined by dynamic light scattering (DLS). Nanoparticles in this size range are well-suited for antimicrobial applications due to their ability to penetrate microbial biofilms, disrupt bacterial membranes, and achieve efficient cellular uptake (82). Sizes under 200 nm are also beneficial for passive penetration into cutaneous and mucosal layers, increasing drug residence time at the site of infection and enhancing overall therapeutic bioavailability. Additionally, uniformity at the nanoscale helps maintain colloidal dispersion and reduces gravitational settling in suspension systems (83).

The zeta potential, recorded at -1.03 ± 0.04 mV, suggests minimal electrostatic stabilization. Although a negative surface charge can offer some repulsive interaction between particles to delay aggregation, this value falls significantly below the ±30 mV threshold commonly associated with robust colloidal stability. This weak surface potential may predispose microemulsion to particle agglomeration or phase separation during storage. Therefore, formulation improvement strategies should be considered such as surface modification with PEG

(PEGylation), chitosan coating, or ionic surfactants to provide either electrostatic or steric stabilization. The inclusion of polymeric stabilizers (e.g., poloxamers or carbomers) could further enhance dispersion uniformity by creating hydration shells or steric hindrance around the nanoparticles, extending shelf-life and minimizing flocculation.

The pH of the formulation, measured at 5.12 ± 0.01, falls within the physiologically acceptable range for topical and mucosal applications. This mildly acidic environment is not only well tolerated by skin and mucosal tissues but may also synergize with silver ion activity to enhance antimicrobial effects. Deviations from this optimal pH range can result in local irritation (84), compromised skin barrier function, or cytotoxicity, particularly when the formulation is applied to inflamed or damaged tissue. Maintaining pH within the 4.5-6.5 range is therefore crucial for both therapeutic efficacy and patient safety.

Further confirmation of silver nanoparticle integrity was provided by UV–visible spectroscopy, which revealed a distinct surface plasmon resonance (SPR) peak at 230 nm. Although classical AgNPs typically exhibit SPR peaks between 400-450 nm, a shift to lower wavelengths can occur in microemulsion systems due to particle size, shape, the surrounding dielectric environment, and nanoparticle surfactant interactions. The observed SPR peak confirms the presence of nanosilver and suggests successful incorporation into the colloidal matrix (85). This optical stability is vital for predicting consistent therapeutic function and validating the reproducibility of the synthetic process.

Despite these favorable findings, the low zeta potential remains a notable limitation, indicating that further formulation optimization is required. Enhancing the stability of the microemulsion may involve systematic adjustments to the oil phase composition, surfactant-to-co-surfactant ratios, and the application of thermodynamic (86) modeling tools (e.g., pseudo-ternary phase diagrams) to identify compositions that resist Ostwald ripening and phase separation. Additionally, the use of lipid-based or hybrid nanocarrier systems, such as nanostructured lipid carriers (NLCs) or solid lipid nanoparticles (SLNs), may offer greater long-term stability while retaining the antimicrobial potency of silver.

In summary, the physicochemical attributes of the AgNPs microemulsion presented in this study namely nanoscale particle size, biocompatible pH, and nanoparticle presence confirmed by SPR are promising for the development of localized antimicrobial therapeutics. However, to ensure clinical viability, further refinement is essential. Future work should prioritize surface engineering of nanoparticles, stabilizer system optimization, and the exploration of scalable, thermodynamically stable delivery platforms. These efforts will be critical for translating this formulation into a robust, patient-friendly product with meaningful antimicrobial efficacy and commercial potential.

2. The Antimicrobial Activity Evaluation

2.1 Antimicrobial susceptibility test by disk diffusion method

The antimicrobial efficacy of the silver microemulsion was evaluated using the disk diffusion method, a standard qualitative assay for assessing microbial susceptibility. The inhibition zones against selected test organisms including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, and *Candida albicans* were measured and are summarized in Table 4.

The silver microemulsion exhibited limited antibacterial activity, with mean inhibition zone diameters of 9.50 mm for *S. aureus*, 9.53 mm for *P. aeruginosa*, and 11.89 mm for *S. mutans*. According to standard interpretive criteria, these zone diameters are indicative of microbial resistance, suggesting that the formulation does not reach the minimum threshold for effective growth inhibition under the conditions of this assay. This limited zone of inhibition may be attributed to the low silver ion release rate, subtherapeutic concentrations at the agar interface, or restricted diffusion of the microemulsion matrix.

No antifungal activity was detected against *C. albicans*, as evidenced by the absence of any measurable inhibition zone, further suggesting that the formulation may lack efficacy against fungal pathogens under current conditions.

These findings highlight the need for formulation optimization to enhance antimicrobial potency, potentially through increasing silver content, modifying release kinetics, or incorporating synergistic agents that improve microbial susceptibility profiles.

Table 4 The Diameter of the growth inhibition zone of silver microemulsion

Pathogens	Diameter of growth inhibition zone (mm)				
	Silver microemulsion	**Micro emulsion**	**Silver nanopaticles**	**0.2%CHX**	**Water**
S. aureus	9.50±0.30	NA	8.96±0.20	13.98±0.13	NA
P. aeruginosa	9.53±0.36	NA	9.17±0.32	10.71±0.45	NA
S. mutans	11.89±0.24	NA	10.68±0.74	17.25±0.59	NA
C. albicans	Colonies within zone	NA	Colonies within zone	15.07±0.96	NA

NA: No activity

The silver microemulsion was resistant to *S. aureus*, *P. aeruginosa* and *S. mutans.* And in antifungal activity, neither the silver microemulsion showed any activities against *C. albicans*. (Figure 4, 5)

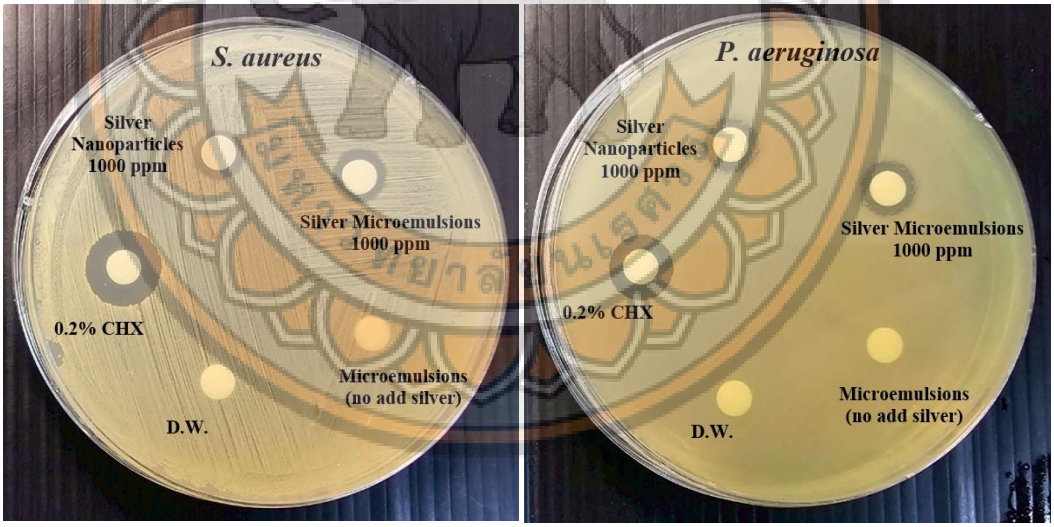

Figure 4 The inhibition zone of silver microemulsion 1000 ppm, microemulsion (no silver nanoparticle), silver nanoparticle 1000 ppm, 0.2% CHX (positive control) and Distilled water (negative control) against *S. aureus* ATCC25923 and *P. aeruginosa* ATCC27853

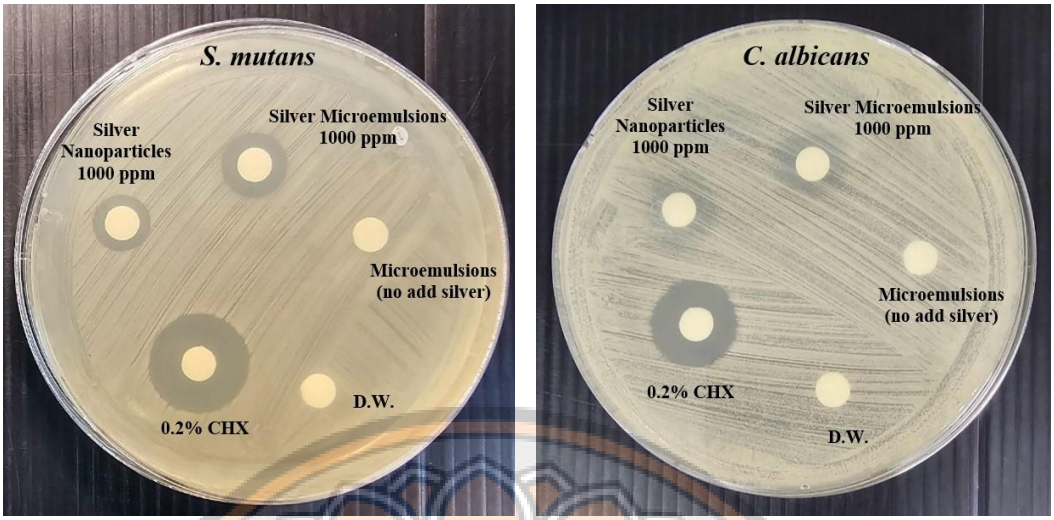

Figure 5 The inhibition zone of silver microemulsion 1000 ppm, microemulsion (no silver nanoparticle), silver nanoparticle 1000 ppm, 0.2% CHX (positive control) and Distilled water (negative control) against *S. mutans* A32-2 and *C. albicans* ATCC10231

The observed resistance of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus mutans* to the silver nanoparticle (AgNPs) microemulsion, along with the complete lack of antifungal activity against *Candida albicans*, is likely attributable to a multifactorial interplay of physicochemical limitations of the formulation and intrinsic microbial defense mechanisms. Silver nanoparticles exert antimicrobial effects predominantly through the controlled release of silver ions (Ag^+), which disrupt bacterial membrane integrity, denature essential proteins, and interfere with nucleic acid function. However, in microemulsion systems, AgNPs are often encapsulated or dispersed within oil or aqueous phases and stabilized by surfactants and co-surfactants, which while beneficial for colloidal stability can significantly hinder the diffusion and release of free silver ions into the surrounding medium. If the release of Ag^+ is too slow, too low in concentration, or physically restricted by the surfactant shell or emulsion interface, the bioactive component may not reach the agar medium in sufficient quantities to exhibit a measurable antimicrobial effect. This problem is compounded in the disk diffusion assay, a method that relies on the passive radial diffusion of antimicrobial agents through a hydrophilic agar matrix.

Because silver microemulsions are typically composed of amphiphilic and often viscous systems, their diffusion through agar is inherently slower and less efficient compared to small hydrophilic drug molecules, thereby underestimating true antimicrobial potential in such static in vitro systems. Moreover, the zeta potential of the microemulsion was measured at -1.03 ± 0.04 mV, a value far below the ±30 mV threshold generally associated with electrostatically stabilized colloids. This weak surface charge could lead to nanoparticle aggregation, reducing the effective surface area and limiting the release of silver ions at the interface with microbial cells. In addition to these physicochemical constraints, the microbial species tested in this study are known for their robust resistance mechanisms. *S. aureus* possesses a thick peptidoglycan layer that can sequester metallic ions and buffer oxidative damage, while *P. aeruginosa* exhibits intrinsic resistance due to its low outer membrane permeability, multiple efflux pumps, and ability to form dense biofilms, which act as physical barriers to nanoparticle penetration. *S. mutans* is likewise protected by an extracellular polysaccharide matrix that can impede nanoparticle access to the cell surface. The complete resistance observed in *C. albicans* is particularly noteworthy, as fungal cells differ significantly from bacterial cells in membrane composition and structural complexity. The cell wall of *C. albicans* is composed of chitin, β-glucans, and mannoproteins, and its plasma membrane contains ergosterol, providing structural robustness and reducing susceptibility to metallic agents like silver. Additionally, *C. albicans* expresses antioxidant enzymes such as superoxide dismutase and catalase that can neutralize reactive oxygen species generated by silver, further diminishing its efficacy. Taken together, these findings suggest that the limited antimicrobial activity of the silver microemulsion arises not from a lack of intrinsic bioactivity of nanosilver, but rather from formulation-dependent barriers that prevent effective release and diffusion, as well as microorganism-specific resistance mechanisms that block or inactivate the action of silver ions. Addressing these limitations will require a multifaceted approach to formulation optimization, including strategies to enhance Ag^{+} ion release (e.g., by modifying the microemulsion interface or using silver salt forms), improving nanoparticle microbe contact (e.g., through charge modification or bioadhesive agents), and evaluating synergistic combinations with other antimicrobial compounds or permeation enhancers. These improvements may enhance the ability of

silver microemulsions to overcome microbial defenses and provide a more reliable antimicrobial effect in both *in vitro* and in *vivo* systems.

Antimicrobial Efficacy Comparison Between Silver Microemulsion and Conventional Silver Nanoparticles

The antimicrobial activity of the silver microemulsion formulation was evaluated in comparison to conventional silver nanoparticles (AgNPs), both tested at an equivalent concentration of 1000 ppm, against three clinically relevant bacterial pathogens: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus mutans*. The disk diffusion method was employed to assess antimicrobial performance, with the diameter of the inhibition zone (ZOI) serving as a surrogate marker of antibacterial potency.

For *S. aureus*, the microemulsion demonstrated a statistically significant improvement in antimicrobial efficacy, producing a larger mean inhibition zone than the conventional AgNPs formulation by 0.54 mm (p = 0.019; 95% CI: 0.22-0.86). A similar trend was observed for *P. aeruginosa*, where the microemulsion also exhibited superior antibacterial activity, with a mean difference of 0.36 mm (p = 0.012; 95% CI: 0.19-0.53). These findings suggest that the microemulsion enhances the bioavailability or diffusion profile of silver, thereby improving antimicrobial activity against both Gram-positive and Gram-negative bacteria.

In the case of *S. mutans*, the microemulsion again produced a larger average zone of inhibition (mean difference = 1.21 mm); however, this difference did not reach statistical significance (p = 0.161; 95% CI: -1.18 to 3.61), indicating comparable efficacy between the two silver formulations for this organism (see Table 5).

From a translational perspective, these results underscore the therapeutic potential of silver microemulsion as a more effective topical antimicrobial agent, particularly in managing infections caused by *S. aureus* and *P. aeruginosa* both of which are frequent culprits in healthcare-associated infections and display rising antimicrobial resistance. The statistically significant improvements observed with the microemulsion formulation support its potential application in wound dressings, surface disinfectants, and antimicrobial coatings on medical devices, where rapid and robust microbial clearance is critical.

Although the difference observed for *S. mutans* did not reach statistical significance, the consistently larger mean inhibition zone indicates that silver microemulsion remains at least as effective as traditional nanoparticles in controlling oral pathogens. These findings suggest a possible role for microemulsion-based silver formulations in dental applications, such as in the prevention of plaque biofilms or management of early-stage periodontal disease, warranting further investigation.

Statistical Analysis

Antimicrobial comparisons were based on the mean diameter of inhibition zones recorded from three independent replicates per microorganism for both the silver microemulsion and silver nanoparticle formulations. For each organism, the difference in mean ZOI between the two treatments was calculated. The standard error of the mean (SEM) of the difference was used to estimate measurement variability. A 95% confidence interval (CI) for the mean difference was derived using the Student's *t*-distribution, which accounts for the small sample size (n = 3 per group). To determine statistical significance, paired *t*-tests were performed for each microorganism. A *p*-value of less than 0.05 was considered statistically significant.

Table 5 Statistical Comparison: Silver Microemulsion vs Silver Nanoparticles

Microorganism	Mean Difference (mm)	SEM	95% CI Lower	95% CI Upper	*p*-value
S. aureus	0.54	0.0755	0.2152	0.8648	0.019
P. aeruginosa	0.36	0.0404	0.1861	0.5339	0.012
S. mutans	1.21	0.5571	-1.1839	3.6105	0.161

Table 5 presents the comparative statistical analysis of antimicrobial efficacy between silver microemulsion and conventional silver nanoparticles against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus mutans*. Using the disk diffusion method, zone of inhibition (ZOI) measurements were analyzed to determine mean differences between the two formulations. For *S. aureus*, the microemulsion produced a significantly larger inhibition zone, with a mean difference of 0.54 mm (SEM =

0.0755, 95% CI: 0.2152–0.8648, p = 0.019), suggesting superior antimicrobial performance. A similar statistically significant improvement was observed for *P. aeruginosa*, with a mean difference of 0.36 mm (SEM = 0.0404, 95% CI: 0.1861-0.5339, p = 0.012). These results confirm that the microemulsion formulation enhances antibacterial efficacy against both Gram-positive and Gram-negative bacteria. In contrast, although the microemulsion yielded a larger average inhibition zone against *S. mutans* (mean difference = 1.21 mm), this result did not reach statistical significance (SEM = 0.5571, 95% CI: –1.1839 to 3.6105, p = 0.161), likely due to high inter-replicate variability. The statistical approach employed a paired *t*-test, which is appropriate given the matched experimental design and small sample size (n = 3 per group), providing control over intra-assay variability. Confidence intervals were used alongside *p*-values to assess both the precision and significance of observed differences. The consistent improvement in antimicrobial activity observed for *S. aureus* and *P. aeruginosa* highlights the pharmacological advantages of the microemulsion system, potentially related to enhanced diffusion, bioavailability, or sustained silver ion release. Meanwhile, the nonsignificant trend for *S. mutans* suggests comparable efficacy with room for further investigation. These findings underscore the therapeutic potential of silver microemulsion formulations in localized antimicrobial applications and justify further development and testing in broader clinical contexts.

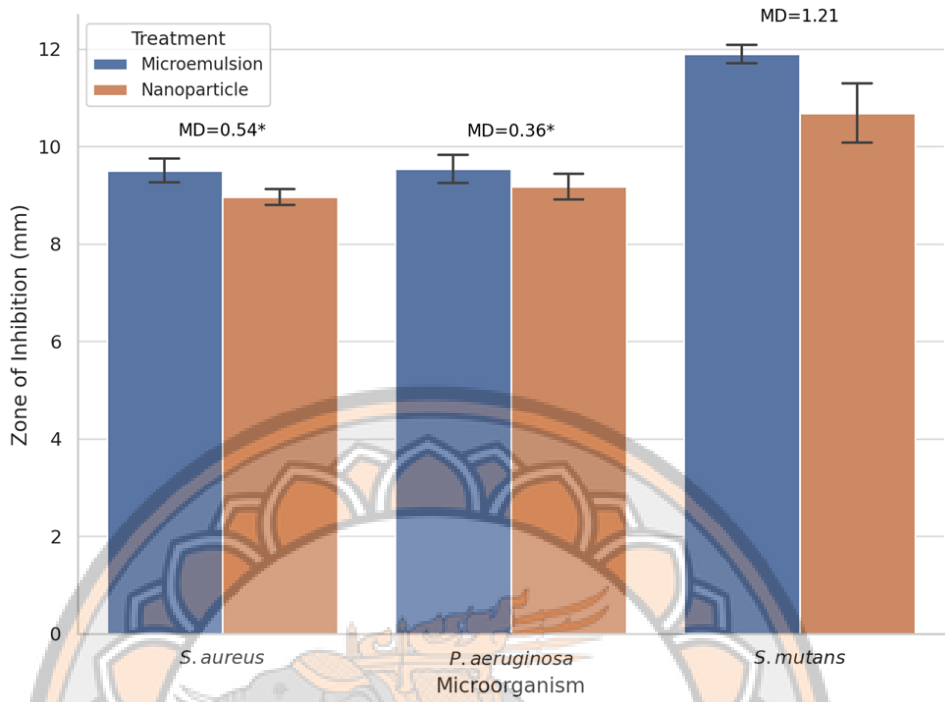

Figure 6 Zone of Inhibition of Silver Microemulsion vs Silver Nanoparticles Against *S. aureus*, P. *aeruginosa*, and *S. mutans*

Note: The bar graph compares the mean zones of inhibition of *S. aureus*, *P. aeruginosa*, and *S. mutans* treated with silver microemulsion and nanoparticle formulations (1000 ppm). Error bars represent standard deviation (n=3). The microemulsion showed significantly greater inhibition than nanoparticles for *S. aureus* (MD = 0.54 mm, $p < 0.05$) and *P. aeruginosa* (MD = 0.36 mm, $p < 0.05$), indicated by an asterisk (*). No significant difference was observed for *S. mutans* (MD = 1.21 mm, $p = 0.161$)

This figure illustrates the comparative antimicrobial activity of silver microemulsion and conventional silver nanoparticles (each at 1000 ppm) against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus mutans*, as assessed by the disk diffusion method. The mean zone of inhibition (ZOI) for each microorganism is presented with standard error bars (n = 3), and mean differences (MD) are annotated above each pair of bars. For *S. aureus*, the microemulsion yielded a significantly larger inhibition zone compared to nanoparticles, with a mean difference of 0.54 mm ($p = 0.019$). A similar significant increase was observed for *P. aeruginosa*, where the microemulsion exceeded the nanoparticle group by 0.36 mm (*p*

= 0.012). For *S. mutans*, the microemulsion exhibited a numerically higher ZOI (MD = 1.21 mm), but the difference was not statistically significant (p = 0.161). These findings demonstrate the enhanced antimicrobial efficacy of the silver microemulsion over conventional nanoparticles against Gram-positive and Gram-negative bacteria, while indicating comparable activity against oral pathogens like *S. mutans*. Asterisks (*) denote statistically significant differences ($p < 0.05$).

2.2 Antimicrobial susceptibility by broth dilution Test

The antimicrobial potency of the silver nanoparticle (AgNPs) microemulsion was further evaluated through determination of its Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) using the standard micro broth dilution method, with concentrations tested up to a maximum of 0.7 mg/mL. These assays provide quantitative benchmarks for assessing the lowest concentration required to inhibit visible bacterial growth (MIC) and to achieve complete bacterial killing (MBC). However, the results indicated suboptimal antimicrobial efficacy, with MIC and MBC values falling near or beyond the highest concentration tested. This suggests that the formulation, in its current state, may have limited bacteriostatic and bactericidal activity against the tested microorganisms.

Several factors may underline this reduced efficacy. One possibility is the inadequate bioavailability of silver ions (Ag^+), which are the primary antimicrobial agents released from AgNPs. In microemulsion systems, AgNPs are often encapsulated or tightly stabilized within the micellar or droplet core, potentially impeding the release of bioactive silver ions into the aqueous environment where bacterial interaction occurs. Additionally, the low zeta potential and broad particle size distribution observed in previous characterization may contribute to nanoparticle aggregation, further reducing the effective surface area and limiting interaction with bacterial membranes. The structural integrity of the microemulsion itself may also act as a diffusional barrier, impairing the penetration of nanoparticles into bacterial cell walls and reducing their ability to exert oxidative or membrane-disruptive damage.

To address these limitations, further formulation optimization is warranted. Increasing the concentration of AgNPs within microemulsion could enhance antimicrobial potency, although care must be taken to maintain biocompatibility. Alternatively,

modifying the stabilizer system, for example, by incorporating surface-active polymers or charge-modifying agents may improve nanoparticle dispersion and facilitate greater Ag^+ release. Another promising strategy would be to integrate synergistic antimicrobial agents, such as essential oils, chlorhexidine, or plant-derived polyphenols, which may disrupt bacterial membranes and enhance silver ion uptake. Such combinatorial approaches could significantly lower the required MIC/MBC values and expand the clinical applicability of the formulation.

In summary, while the current AgNPs microemulsion exhibits measurable antimicrobial properties, its bactericidal efficiency remains subtherapeutic at the tested concentrations. These findings underscore the need for rational formulation engineering to optimize silver delivery, enhance microbial susceptibility, and realize the full therapeutic potential of AgNPs microemulsions for localized infection control. Detailed MIC and MBC values for each organism are presented in Table 6, which provides critical reference data for future formulation refinement.

Table 6 The evaluation of MIC and MBC

Pathogens	MIC mg/mL	MBC/ MFC mg/mL
1. *Staphylococcus aureus*	0.4	0.5
2. *Pseudomonas aeruginosa*	0.3	0.4
3. *Streptococcus mutans*	0.2	0.3
4. *Candida albicans*	0.4	0.7

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) results further reinforce the limitations of the current silver nanoparticle (AgNPs) microemulsion formulation, as it failed to achieve complete bacterial eradication at clinically relevant concentrations (MIC > 0.7 mg/mL). This diminished antimicrobial activity may be attributed to restricted silver ion (Ag^+) release resulting from encapsulation within the microemulsion matrix, which likely limits both bioavailability and direct interaction with bacterial membranes (87). Prior studies have demonstrated that AgNPs exert their bactericidal effects primarily through mechanisms involving direct membrane disruption, generation of reactive

oxygen species (ROS), and sustained release of silver ions (87). However, the structural composition of the microemulsion used in this study may have impeded these mechanisms, thereby reducing overall antimicrobial efficacy.

To enhance the formulation's bactericidal performance, several rational strategies can be employed. One approach involves modifying the nanoparticle surface using cationic coatings, such as chitosan or polyethyleneimine, which have been shown to enhance electrostatic interactions with negatively charged bacterial cell walls, thereby promoting nanoparticle adhesion and membrane penetration (87). Additionally, optimization of the surfactant composition within the microemulsion could improve nanoparticle dispersion and facilitate more efficient Ag^+ ion release (87). Another promising strategy is the incorporation of synergistic antimicrobial agents such as essential oils, antimicrobial peptides, or bioactive polymers which have been reported to enhance AgNPs penetration and augment bactericidal activity (87-89). This is supported by growing evidence from nanotechnology-based antimicrobial research, which shows that hybrid formulations combining AgNPs with secondary bioactive compounds exhibit improved efficacy against biofilms and multidrug-resistant pathogens (86).

Future investigations should prioritize mechanistic studies including bacterial membrane integrity assays, biofilm disruption models, and time-kill kinetics to better elucidate the mode of action of the AgNPs microemulsion and guide targeted formulation refinement. Furthermore, in vivo studies will be essential to evaluate pharmacokinetics, tissue compatibility, and real-world therapeutic performance in localized infection models (87-89). Collectively, these findings underscore the importance of formulation optimization in the development of AgNPs-based antimicrobial agents and highlight the need for targeted strategies to improve bacterial interactions, increase silver ion release, and enhance overall therapeutic potential (88, 89).

3. Evaluation of the Cytotoxicity Testing

The cytotoxic profile of the silver nanoparticle (AgNPs) microemulsion was evaluated using the MTT assay in L929 murine fibroblast cell lines, a widely accepted model for assessing biocompatibility of topical and implantable formulations. The

assay was designed to quantify mitochondrial metabolic activity as an indirect measure of cell viability following exposure to increasing concentrations of the AgNPs microemulsion. The results demonstrated that at concentrations below 16 µg/mL, the formulation preserved greater than 70% cell viability, meeting the threshold for acceptable biocompatibility as defined by ISO 10993-5 standards. This suggests that microemulsion, at sub-therapeutic or localized concentrations, exhibits minimal cytotoxicity and may be safe for further development in topical or wound-healing applications.

However, exposure to higher concentrations particularly at 32 µg/mL resulted in a marked reduction in cell viability, accompanied by visible morphological alterations in fibroblast cells, including cellular shrinkage, cytoplasmic condensation, and detachment from the culture substrate. These findings are indicative of dose-dependent cytotoxicity, which may be mediated by increased intracellular oxidative stress, disruption of membrane integrity, or mitochondrial dysfunction at higher AgNPs levels. Such responses are consistent with previously reported mechanisms of AgNPs-induced cytotoxicity, where nanoparticle internalization leads to reactive oxygen species (ROS) generation and subsequent activation of apoptotic pathways. Overall, these results underscore the importance of dose optimization in the therapeutic application of AgNPs microemulsions. While the formulation demonstrates a favorable safety margin at lower concentrations, exceeding cytotoxic thresholds may compromise tissue compatibility. Table 7 summarizes the viability data across tested concentrations, highlighting the critical balance between antimicrobial efficacy and cellular safety in the context of localized therapeutic delivery.

Table 7 Cytotoxicity Assessment of Silver Microemulsion in L929 Murine Fibroblast Cells Using MTT Assay

Silver Microemulsion Concentration (µg/mL)	Cell Viability (%)
0.5 – 16 µg/mL	> 70% (Non-toxic)
32 µg/mL	Cytotoxic

Table 7 summarizes the cytotoxicity findings from the MTT assay performed on L929 murine fibroblast cells exposed to varying concentrations of silver nanoparticle (AgNPs) microemulsion. At concentrations ranging from 0.5 to 16 µg/mL, cell viability consistently remained above 70%, classifying the formulation as non-cytotoxic according to ISO 10993-5 guidelines, which set a 70% viability threshold for biocompatibility. This indicates that the microemulsion is well tolerated by fibroblast cells at these concentrations and is likely to be safe for localized biomedical applications such as wound dressings or topical antimicrobial therapies. However, exposure to a higher concentration of 32 µg/mL resulted in a significant reduction in cell viability, categorizing the formulation as cytotoxic at this dose. The sharp decline suggests the onset of dose-dependent cellular stress, potentially linked to mechanisms such as silver ion-induced oxidative damage, mitochondrial dysfunction, or membrane disruption. These findings highlight the critical importance of dosage control in the design and clinical application of AgNPs-based therapies, ensuring antimicrobial efficacy is achieved without compromising host tissue viability.

To evaluate the cytotoxicity effect of silver microemulsion on L929 cells viability, cells were exposed to different concentrations of silver microemulsion 0.5, 1, 2, 4, 8, 16, 32 µg/mL, for 24 hours. The cytotoxicity of the L929 cell lines was detected using an MTT assay. (Figure 7)

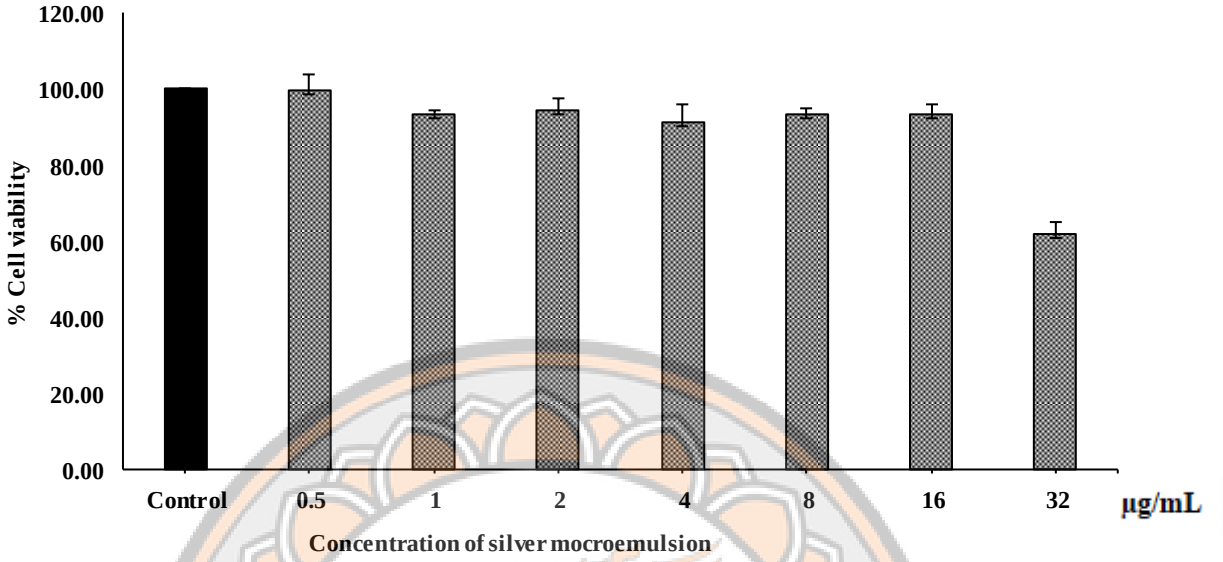

Figure 7 Viability of L929 cell lines after exposure to silver microemulsion for 24h (control-no treated silver microemulsion)

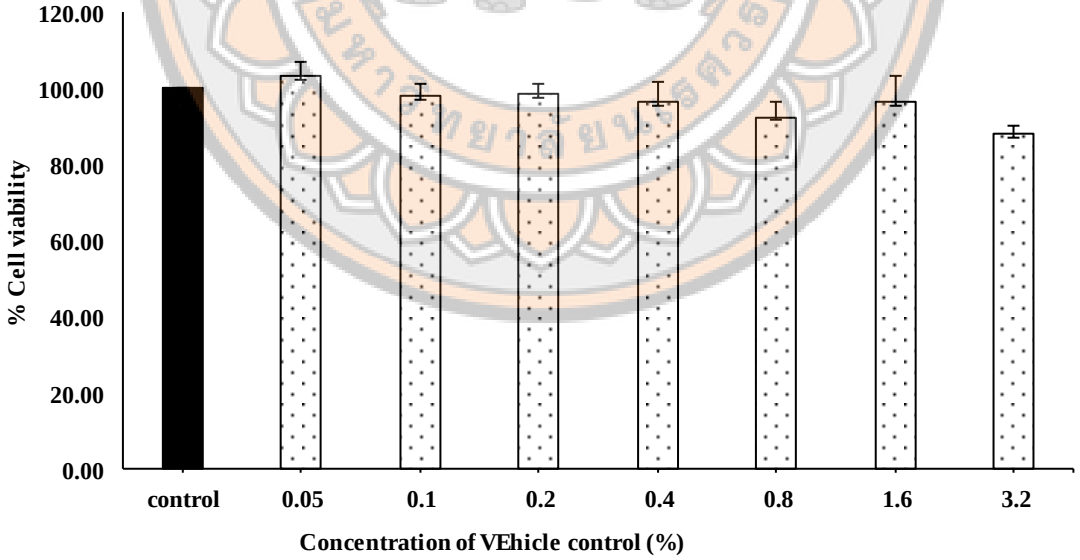

Figure 8 Viability of L929 cell lines after exposure to vehicle control (microemulsion no add silver nanoparticle) and control-no treated microemulsion

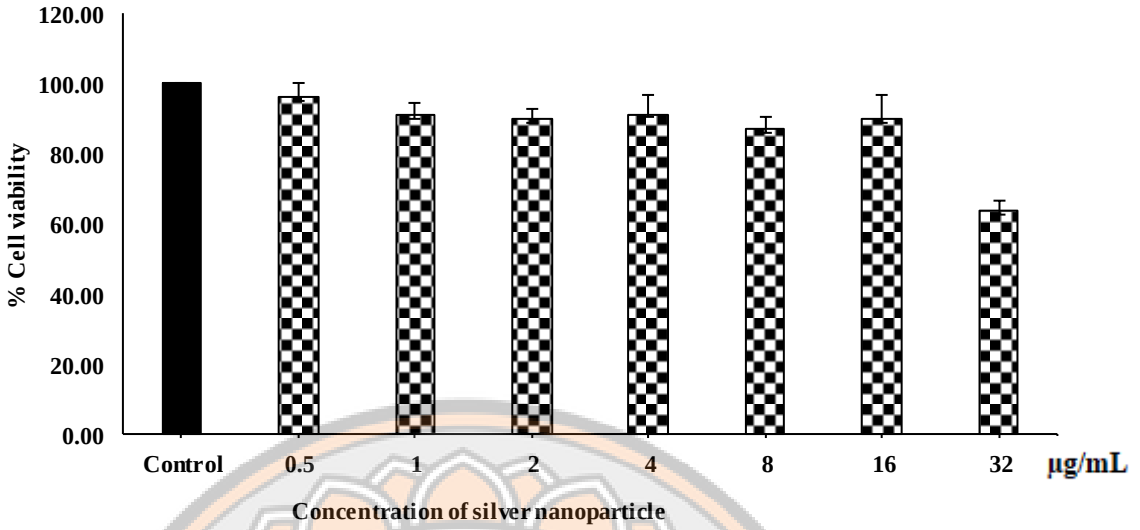

Figure 9 Viability of L929 cell lines after exposure to silver nanoparticle for 24h (Control-No treated silver nanoparticle)

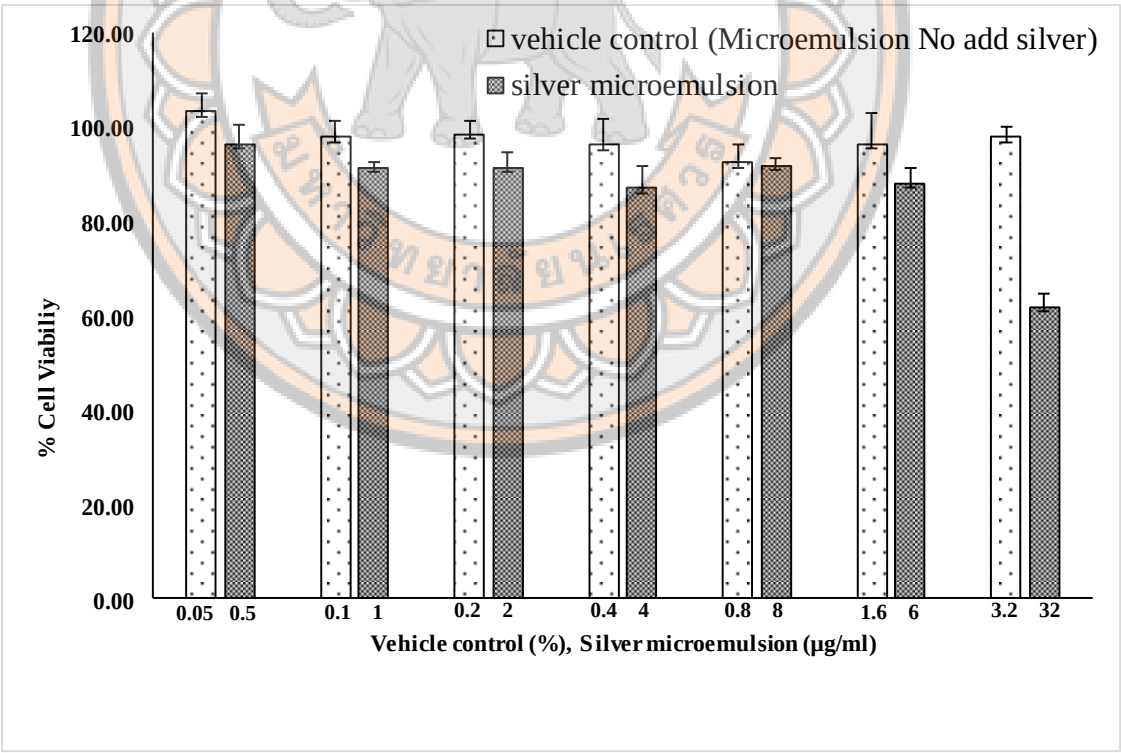

Figure 10 The Viability rate was detected by MTT assay in L929 cells cultured to compared between silver microemulsion and vehicle control (Microemulsion no add silver nanoparticle) after 24h of incubation

These findings indicate that the silver microemulsion formulation possesses a well-defined therapeutic window, with concentrations up to 16 µg/mL demonstrating acceptable biocompatibility in L929 fibroblast cells, whereas higher doses such as 32 µg/mL are associated with significant cytotoxic effects. This highlights the critical importance of dose optimization in ensuring both safety and efficacy, particularly for formulations designed for localized antimicrobial delivery, where prolonged tissue exposure is expected. The observed threshold for cytotoxicity underscores the need for precise control over nanoparticle concentration and release kinetics in clinical use. Future investigations should prioritize formulation refinement, including strategies to improve nanoparticle stabilization, control silver ion release, and enhance tissue targeting. Such approaches will be essential for maximizing antimicrobial performance while minimizing host cell toxicity, thereby supporting the safe and effective translation of silver microemulsion systems into therapeutic applications

Limitations of the Study

Despite providing valuable insights, this study has several limitations that warrant consideration. First, the antimicrobial assays were conducted exclusively in vitro, which limits the extrapolation of findings to complex in vivo environments. The disk diffusion and broth dilution methods may underestimate antimicrobial efficacy due to poor diffusion properties of the microemulsion matrix in agar-based systems, especially given the amphiphilic nature of the formulation. Moreover, the absence of biofilm models fails to reflect clinical challenges where bacterial communities are embedded in protective extracellular matrices, particularly for pathogens such as *S. mutans* and *P. aeruginosa*.

Second, the zeta potential value (-1.03 mV) indicates poor colloidal stability, suggesting that nanoparticle aggregation may have occurred during testing, potentially compromising silver ion release and antimicrobial interaction. This physicochemical constraint may confound efficacy measurements and warrants further optimization. Additionally, cytotoxicity assessment was limited to a single fibroblast cell line (L929) and a 24-hour exposure period. This narrow evaluation may not fully capture long-term or tissue-specific toxicological responses, which are critical for real-world topical or mucosal applications.

Third, only three bacterial species and one fungal strain were evaluated, which does not represent the full spectrum of clinically relevant pathogens. The lack of resistant strains (e.g., MRSA, ESBL-producing *P. aeruginosa*) also limits the understanding of this formulation's applicability in treating multidrug-resistant infections. Lastly, the formulation was tested at fixed concentrations, and no time-kill kinetics, post-antibiotic effect (PAE), or mechanistic studies (e.g., ROS generation, membrane integrity assays) were conducted to elucidate the dynamics and pathways of antimicrobial action.

Implications for Clinical Practice

Despite its limitations, this study lays the groundwork for the development of silver microemulsion-based antimicrobials with localized application potential, particularly in wound care, dental plaque control, surgical dressing, and mucosal antisepsis. The formulation demonstrated adequate biocompatibility at concentrations ≤16 μg/mL, supporting its safety profile for superficial clinical applications where systemic exposure is minimal. The enhanced antibacterial effect against *S. aureus* and *P. aeruginosa* both frequent agents in healthcare-associated infections suggests that silver microemulsions could serve as an adjunct or alternative to conventional antiseptics such as chlorhexidine, especially in patients with known hypersensitivity or cytotoxic reactions.

Importantly, the observed narrow therapeutic window necessitates cautious dosing and reinforces the importance of controlled-release mechanisms in any future commercial product. The lack of antifungal activity against *C. albicans* also highlights that silver microemulsions may be best suited for bacterial infections, and not for broad-spectrum use without further modifications. For practical integration, this formulation could be adapted into hydrogel dressings, sprays, or coated materials where sustained antimicrobial action at the interface is desired.

Future Research Directions and Innovation Opportunities

To advance toward clinical translation, several research and innovation avenues must be pursued. First, formulation optimization is essential. This includes enhancing nanoparticle dispersion and stability through charge modulation (e.g., PEGylation, chitosan coating) and improving silver ion release via microemulsion interface engineering or triggered-release platforms (e.g., pH-sensitive or

temperature-responsive systems). Combining AgNPs with synergistic antimicrobial agents such as essential oils, antimicrobial peptides, or nitric oxide donors may increase efficacy against resistant strains and biofilms while minimizing the required silver dose.

Second, future studies should incorporate comprehensive mechanistic analyses, including ROS quantification, membrane permeability assays, and gene expression profiling to understand how AgNPs act within the microemulsion framework. Additionally, biofilm and 3D tissue culture models are needed to evaluate antimicrobial penetration and persistence in complex biological environments. Most importantly, in vivo studies using validated animal models of infected wounds or oral mucosal lesions will be critical to confirm pharmacokinetics, therapeutic efficacy, and safety in a physiological context.

On the innovation front, this platform opens avenues for developing customizable, patient-specific antimicrobial coatings, especially for medical devices or dental applications. A modular design approach could allow for the co-delivery of anti-inflammatory or wound-healing agents, making micro-emulsion a multifunctional therapeutic system. Additionally, exploring scalable manufacturing techniques, such as microfluidics or spray-drying, could enhance commercial feasibility.

CHAPTER V

CONCLUSIONS

This study comprehensively evaluated a novel silver nanoparticle (AgNPs) microemulsion formulation with respect to its physicochemical characteristics, antimicrobial efficacy, and cytotoxicity profile. The results demonstrated that the formulation achieved favorable nanoparticle distribution (mean diameter ≈ 175.63 nm) and optical confirmation of AgNPs presence via surface plasmon resonance analysis, indicating successful nanoparticle incorporation. Although the zeta potential was relatively low (-1.03 mV), suggesting limited colloidal stability, the formulation remained macroscopically uniform and functionally active under short-term testing conditions.

The antimicrobial assays revealed that the silver microemulsion exhibited statistically significant efficacy against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, surpassing conventional AgNPs formulations in inhibition zone diameter, albeit remaining less effective than chlorhexidine. For *Streptococcus mutans*, the results suggested non-inferiority but lacked statistical significance. Notably, no antifungal activity was observed against *Candida albicans*, indicating a narrow antibacterial spectrum. Minimum inhibitory concentration (MIC) data further confirmed reduced potency, with incomplete bacterial inhibition at concentrations up to 0.7 mg/mL, highlighting the need for enhanced nanoparticle bioavailability and membrane interaction.

Cytotoxicity testing using the MTT assay in L929 murine fibroblast cells revealed a clear therapeutic window: concentrations ≤16 µg/mL maintained >70% cell viability, while concentrations ≥32 µg/mL demonstrated marked cytotoxic effects and morphological changes. This emphasizes the importance of precise dose control in clinical applications.

Despite these strengths, the study has limitations. All assays were performed in vitro without validation in biofilm or in vivo models, and mechanistic data (e.g., ROS, membrane integrity) were not captured. The scope of microbial testing was narrow, and comparative toxicity data with free AgNPs were not included. These

constraints limit the generalizability of findings and the mechanistic understanding of action and safety.

From a translational perspective, the silver microemulsion holds promise as a localized antimicrobial therapy, particularly in wound care, oral hygiene products, and surface antisepsis. Its enhanced performance over conventional nanoparticles at lower toxicity thresholds supports its further development. However, the absence of antifungal action and the limited spectrum of antibacterial activity call for formulation improvement.

Future innovations may involve:

- Surface modification (e.g., cationic polymers or PEGylation) to enhance nanoparticle bacteria interaction and reduce aggregation.
- Co-delivery with synergistic agents such as antimicrobial peptides, essential oils, or nitric oxide donors to expand the spectrum and efficacy.
- Development of controlled-release systems (e.g., hydrogel matrices, responsive nanocarriers) to sustain local delivery while minimizing cytotoxic risk.

Recommended future research includes:

1. Mechanistic studies to evaluate silver ion release, ROS generation, and bacterial membrane disruption.
2. *In vivo* validation in wound and mucosal infection models to assess therapeutic efficacy, pharmacokinetics, and biocompatibility.
3. Comparative safety profiling between microemulsion-encapsulated and unformulated AgNPs.
4. Scale-up and stability testing for industrial and clinical feasibility.

In summary, this research supports the potential of silver microemulsions as a safe and effective platform for localized antibacterial treatment. However, clinical translation will require continued formulation refinement, comprehensive toxicological validation, and mechanistic clarification to realize their full therapeutic utility.

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APPENDIX

Results

		Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 21.77	Peak 1:	26.02	89.1	15.41
PdI: 0.340	Peak 2:	369.6	10.9	222.6
Intercept: 0.931	Peak 3:	0.000	0.0	0.000
Result quality Good				

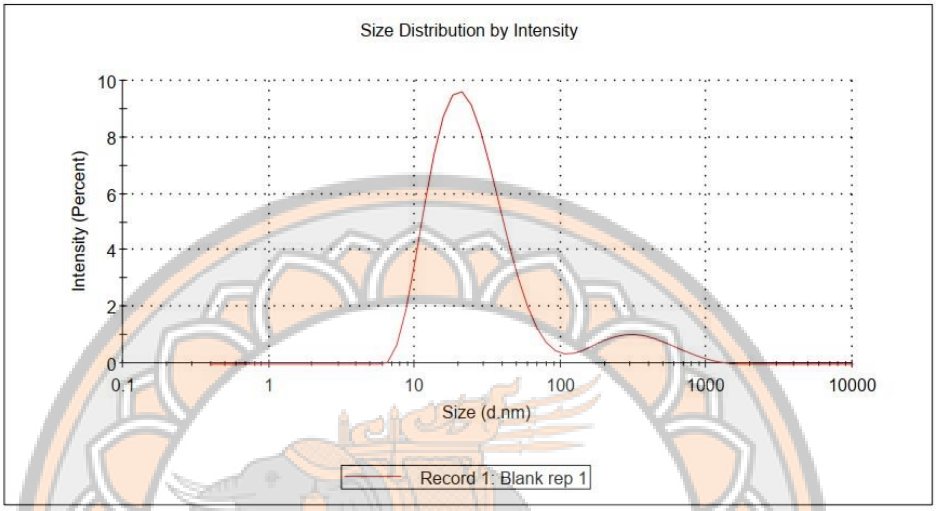

Figure 10 Size distribution by intensity analysis of Microemulsion (n=1)

Results

		Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 21.55	Peak 1:	25.93	89.8	14.56
PdI: 0.337	Peak 2:	550.2	10.2	310.5
Intercept: 0.930	Peak 3:	0.000	0.0	0.000
Result quality Good				

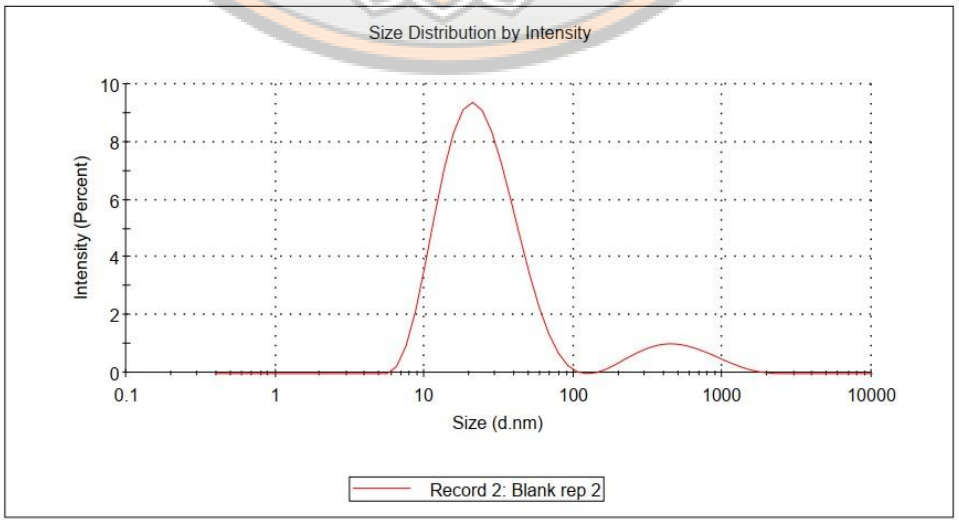

Figure 11 Size distribution by intensity analysis of Microemulsion (n=2)

Results

			Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	21.64	Peak 1:	26.69	90.1	15.16
PdI:	0.343	Peak 2:	580.2	9.9	343.3
Intercept:	0.931	Peak 3:	0.000	0.0	0.000
Result quality	Good				

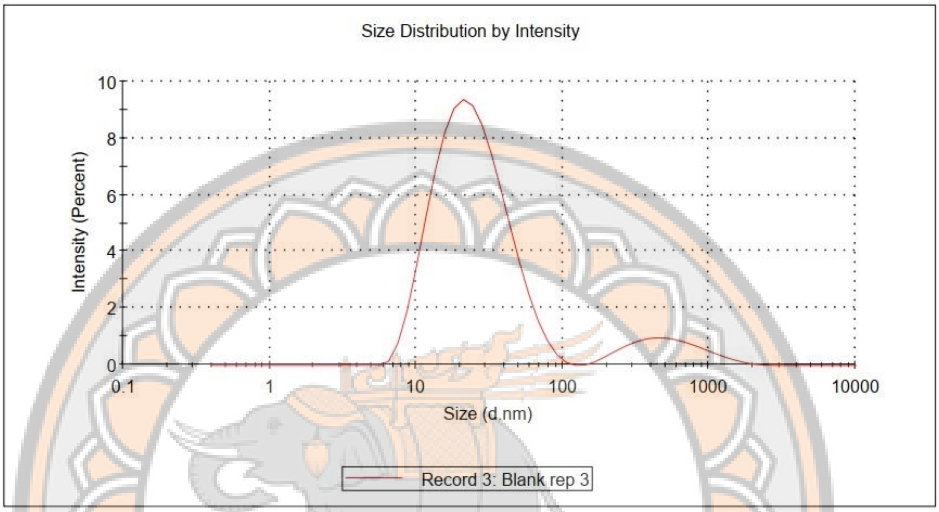

Figure 12 Size distribution by intensity analysis of Microemulsion (n=3)

Results

			Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	175.7	Peak 1:	315.1	91.4	135.6
PdI:	0.576	Peak 2:	16.51	8.6	4.137
Intercept:	0.965	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

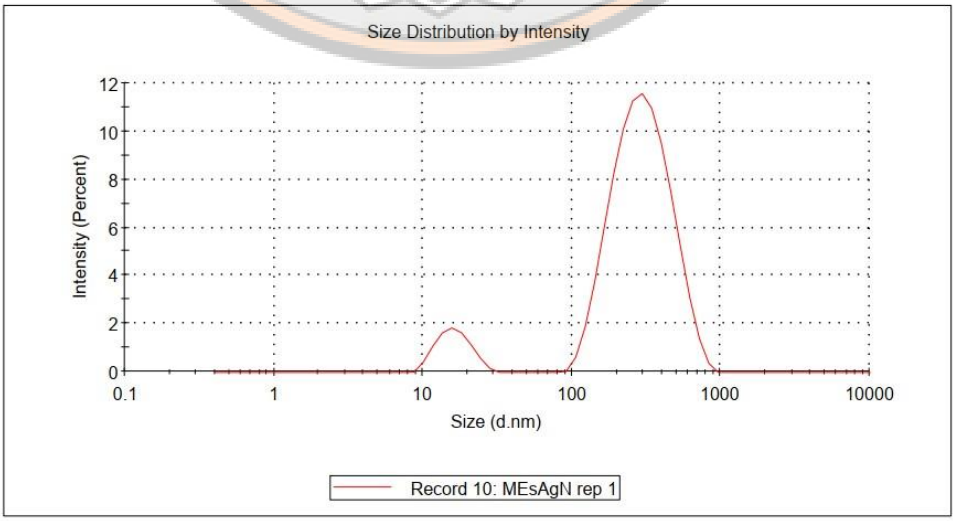

Figure 13 Size distribution by intensity analysis of Silver Microemulsion (n=1)

Results

			Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	176.3	Peak 1:	309.2	90.5	105.2
PdI:	0.582	Peak 2:	18.41	9.5	6.567
Intercept:	0.965	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

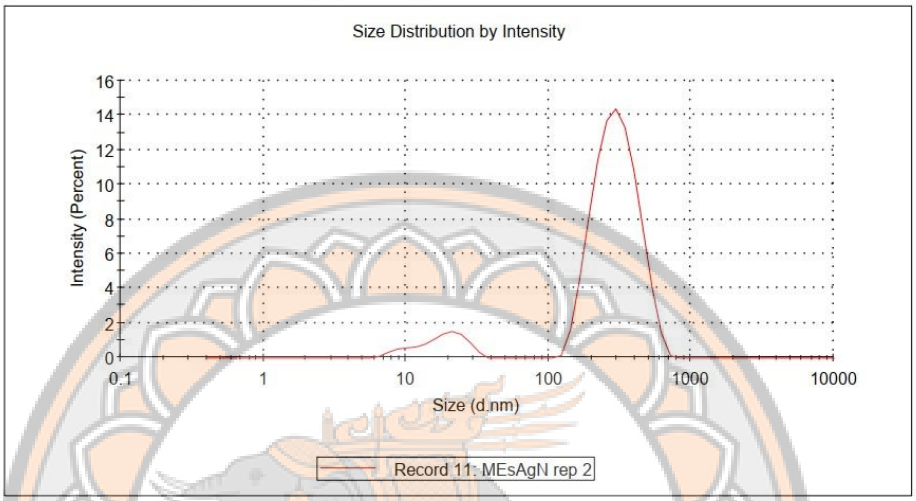

Figure 14 Size distribution by intensity analysis of Silver Microemulsion (n=2)

Results

			Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	175.9	Peak 1:	304.4	90.3	101.3
PdI:	0.573	Peak 2:	19.67	8.2	4.963
Intercept:	0.961	Peak 3:	7.771	1.5	1.370
Result quality	Refer to quality report				

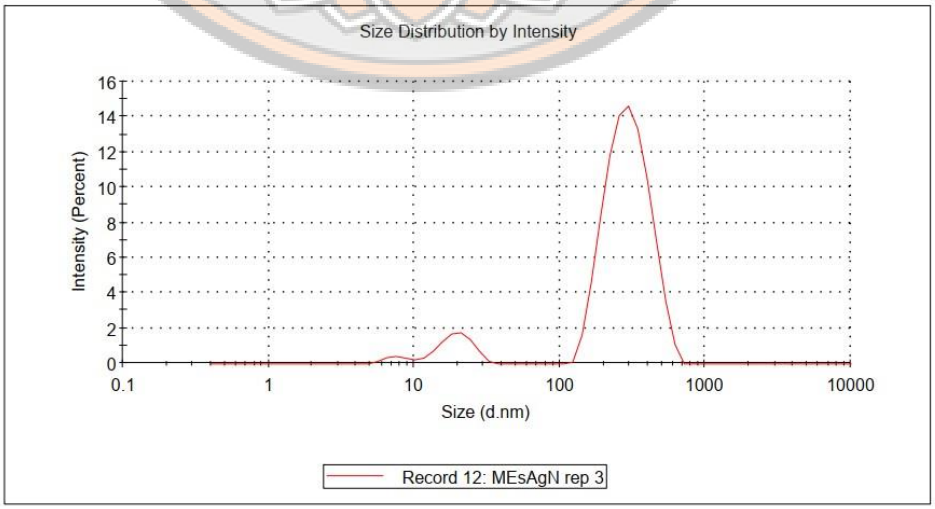

Figure 15 Size distribution by intensity analysis of Silver Microemulsion (n=3)

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