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Fabrication, Optimization, and Pharmacological Assessment of Apigenin-Loaded Nanoemulsion Gels: A Novel Topical Approach for the Treatment Urinary Tract Infections

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ABSTRACT

The present study focused on the formulation, characterization, and evaluation of a nanoemulsion-based gel incorporating apigenin, chitosan, and eucalyptus oil for enhanced drug delivery and antibacterial activity. Three formulations—blank nanoemulsion, chitosan-based nanoemulsion gel, and eucalyptus oil nanoemulsion—were developed and subjected to physicochemical characterization and thermodynamic stability testing. The formulations exhibited optimal droplet size (59.54–87.48 nm), zeta potential (-22.4 to +27.2 mV), and polydispersity index (0.119–0.338), ensuring colloidal stability. FTIR analysis confirmed successful drug incorporation without significant chemical interactions. The drug content was 95.80 ± 1.26% for the nanoemulsion and 93.75 ± 1.30% for the nanoemulsion gel, indicating efficient entrapment. The in vitro drug release study demonstrated a sustained release profile, with NEMULF2 (nanoemulsion) releasing 81.56% and NEMULGF3 (nanoemulsion gel) releasing 71.87% over 12 hours, confirming the gel's controlled-release properties. The antibacterial study revealed a concentration-dependent inhibition against Pseudomonas aeruginosa (24.86 mm at 500 µg/mL) and Escherichia coli (26.65 mm at 500 µg/mL), significantly surpassing the pure drug's efficacy. The findings suggest that nanoemulsion-based gels enhance drug stability, bioavailability, and antimicrobial efficacy, making them promising candidates for topical therapeutic applications.

Keywords: *Nanoemulsion, Nanogel, Antibacterial, Uropathogens, Apigenin, UTIs.*

1. INTRODUCTION

Nanoemulsions are advanced colloidal drug delivery systems that have gained significant attention due to their ability to enhance the solubility, stability, and bioavailability of both hydrophobic and hydrophilic drugs. These systems are composed of oil, surfactants, and an aqueous phase, forming nano-sized droplets typically ranging from 20 to 200 nm in diameter. The small droplet size provides a large surface area, facilitating rapid drug absorption and efficient permeation across biological membranes. Due to their ability to prevent drug precipitation and protect active pharmaceutical ingredients (APIs) from

degradation, nanoemulsions are particularly beneficial for poorly water-soluble drugs, offering enhanced bioavailability and therapeutic efficacy (1-3). In addition to their superior drug-loading capacity, nanoemulsions provide a targeted and sustained drug release profile, ensuring controlled drug delivery over an extended period. The choice of oil plays a crucial role in drug solubilization, while surfactants and co-surfactants stabilize the formulation, reducing interfacial tension and preventing phase separation. Due to their ability to penetrate the skin barrier efficiently, nanoemulsions have been widely explored for topical, transdermal, and mucosal applications, offering advantages such as reduced irritation, enhanced permeation, and site-specific drug delivery. Despite their advantages, nanoemulsions often suffer from low viscosity, making them difficult to apply topically without a suitable carrier. To overcome this limitation, nanoemulsion-based gels are developed by incorporating a gelling agent, which enhances viscosity, adhesion, and spreadability for improved patient compliance. These gels maintain the stability of nano-sized droplets while preventing phase separation, thereby extending drug release and enhancing therapeutic effects. The incorporation of polymers such as chitosan or carbopol into nanoemulsions results in a semi-solid formulation that ensures prolonged retention at the site of application, making nanoemulsion-based gels an excellent choice for topical and transdermal drug delivery (4-6). Overall, nanoemulsion-based gels combine the advantages of nanoemulsions and gel formulations, providing enhanced solubility, stability, and prolonged drug action. Their unique structural and physicochemical properties make them highly suitable for dermatological, antimicrobial, and pain management applications, offering an efficient and patient-friendly alternative to conventional drug delivery systems.

Apigenin, a naturally occurring flavonoid, possesses anti-inflammatory, antioxidant, and antibacterial properties, but its poor water solubility and low bioavailability limit its therapeutic potential. To overcome these limitations, nanoemulsion technology has been employed to enhance apigenin's absorption and effectiveness (Kaur et al., 2017, Kaur et al., 2019, Smoleński et al., 2021. Eucalyptus oil, a well-known essential oil with antimicrobial activity, is incorporated into the formulation to synergistically enhance the antibacterial effects of apigenin. Chitosan, a biodegradable and biocompatible polymer, is used as a gelling agent to improve formulation stability and adhesion for prolonged therapeutic effects (7, 8). This study aims to develop and evaluate nanoemulsion-based gel formulations of apigenin with eucalyptus oil and chitosan for topical drug delivery. The formulations were characterized for droplet size, zeta potential, polydispersity index, drug content, and thermodynamic stability. Furthermore, in vitro drug release and antibacterial activity against uropathogenic bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) were assessed. The study provides valuable insights into the potential of nanoemulsion-based gels for enhanced topical and antimicrobial applications.

2. MATERIAL AND METHODS

Drugs and chemicals:

Apigenin was obtained as a generous gift sample from Kemler Lifesciences, Karnal, India, ensuring its authenticity and purity for the study. Xanthan gum, a crucial polymer in the formulation, was procured from Merck, India, which is known for its high-quality chemical supplies. Chitosan, a biopolymer with mucoadhesive and controlled drug release properties, was purchased from Loba Chemical company, Mumbai, India, a globally recognized supplier of research-grade chemicals. All other chemicals and reagents required for the study were sourced exclusively from reliable and verified suppliers, ensuring consistency and reproducibility of the experimental results. Additionally, every chemical and reagent utilized was of analytical grade, meeting the necessary standards for precision and accuracy in pharmaceutical research.

Preparation of nanoemulsion formulation (NEF):

A previously published method was utilised fr the fabrication of the formulations (9) with slight modifications. The nanoemulsion formulations were prepared using a spontaneous emulsification method. Accurately weighed quantities of eucalyptus oil, xanthan gum, and Tween-80 were taken according to the composition detailed in Table 1. Initially, eucalyptus oil was mixed with the surfactant Tween-80 under continuous stirring to ensure uniform dispersion. Xanthan gum was gradually added to this mixture to achieve a stable emulsion consistency. For formulations containing the drug, the required amount of drug (100 mg) was dissolved in eucalyptus oil before the addition of the surfactant. The prepared oil phase was then slowly added to distilled water under constant stirring at 800–1000 rpm using a magnetic stirrer to facilitate emulsification. Stirring was continued for 30 minutes to ensure proper dispersion and homogeneity of the nanoemulsion. The obtained nanoemulsions were allowed to equilibrate at room temperature for 24 hours to ensure stability. The blank formulation (NEMULF-1) was prepared following the same procedure but without the addition of the drug. The final formulations were visually inspected for phase separation, and their physical characteristics, including droplet size and stability, were further analyzed for evaluation. Table 1 displays the compositions for the formulations.

Table 1. Composition of the formulation for nanoemulsion formulations with all component concentrations

Code for the formulations	Drug (w/w)	Xanthan gum (w/w)	Eucalyptus oil w/w	Tween-80	Distilled water Q. S to make 100 g
NEMULF-1 (Blank)	-	18 g	20 g	15 g	47 g

NEMULF-2	100 mg	18 g	20 g	15 g	46.9 g
NEMULF-3	100 mg	16 g	23 g	14 g	46.9 g
NEMULF-4	100 mg	21 g	18 g	13 g	47.9 g

Evaluation of nanoemulsions:

The prepared nanoemulsions were evaluated using various physicochemical and stability parameters to ensure their suitability for pharmaceutical applications. Visual inspection was conducted to assess homogeneity, transparency, and phase separation, noting any signs of creaming, sedimentation, or coalescence to determine physical stability. The droplet size and polydispersity index (PDI) were measured using dynamic light scattering (DLS), with a low PDI value indicating monodispersity. Zeta potential analysis was performed to evaluate the surface charge of the nanoemulsion droplets, where a high absolute value (greater than ±30 mV) suggested good stability due to electrostatic repulsion, preventing droplet aggregation. The pH of the formulations was measured using a digital pH meter, ensuring compatibility with biological systems by maintaining it within the acceptable range of 5.5 to 7.5 to prevent irritation. Viscosity analysis was carried out using a rotational viscometer at room temperature to characterize the rheological behavior, distinguishing between Newtonian and non-Newtonian flow properties. The refractive index was determined using a refractometer to confirm the isotropic nature of the nanoemulsions, a key characteristic of stable formulations. Drug content estimation was performed using a UV-visible spectrophotometer or high-performance liquid chromatography (HPLC) to determine the percentage of drug present in the formulation compared to the theoretical amount. In vitro drug release studies were conducted using the dialysis membrane method, wherein the nanoemulsions were placed in dialysis bags and immersed in a dissolution medium under constant stirring, with aliquots withdrawn at regular intervals for spectrophotometric analysis. Stability studies were carried out under various storage conditions, including refrigeration at 4°C, room temperature at 25°C, and accelerated conditions at 40°C ± 2°C with 75% RH, monitoring changes in physical appearance, droplet size, zeta potential, and drug content over time. Transmission Electron Microscopy (TEM) analysis was performed to visualize the morphology and structural integrity of the nanoemulsion droplets, providing insights into their spherical nature and uniformity..

Preparation of chitosan gel:

The chitosan gel was prepared using the dispersion method to ensure uniform gel formation. A weighed amount of chitosan was gradually added to an aqueous acetic acid solution (1% v/v) under continuous stirring to facilitate its dissolution. The mixture was stirred at room temperature for 3–4 hours until a clear and homogeneous solution was obtained. To enhance the gel's structural integrity and stability, a crosslinking agent such as glycerol or polyethylene glycol was incorporated at a predetermined concentration, followed by continuous stirring. Once the chitosan solution was well dispersed, the required amount of active drug or bioactive compound was added with gentle mixing to achieve uniform distribution within the gel matrix. The pH of the formulation was adjusted to a physiologically acceptable range (typically 5.0–6.5) using sodium hydroxide or hydrochloric acid, ensuring compatibility with biological systems. The final formulation was subjected to mild sonication to remove any entrapped air bubbles, ensuring a smooth and homogeneous gel consistency. The prepared chitosan gel was then transferred to sterilized containers and stored under appropriate conditions for further evaluation of its physicochemical properties and stability.

Preparation of nanoemulsion gel (NEG):

The nanoemulsion gel was prepared by incorporating the optimized nanoemulsion into a suitable gel base to enhance its topical application properties. Initially, the nanoemulsion was prepared using the spontaneous emulsification method, where eucalyptus oil, surfactant (Tween-80), and xanthan gum were mixed under continuous stirring. The drug was dissolved in the oil phase before adding it to the aqueous phase, followed by high-speed homogenization to achieve a stable nanoemulsion. For gel preparation, an appropriate gelling agent, xanthan gum, was dispersed in distilled water with continuous stirring and allowed to swell for a predetermined time to ensure complete hydration. The nanoemulsion was then slowly incorporated into the gel base under gentle stirring to maintain homogeneity and avoid phase separation. The pH of the formulation was adjusted to a physiologically acceptable range (5.5–6.5) using a dilute sodium hydroxide solution. The final nanoemulsion gel was sonicated to remove air bubbles, ensuring a smooth and uniform consistency. The prepared formulation was stored in suitable containers for further evaluation, including spreadability, viscosity, drug content, and stability studies.

Characterizations:

Thermodynamic stability and Heat cooling cycle:

The thermodynamic stability of the nanoemulsions was evaluated through the heat-cooling cycle, where formulations were alternately stored at 4°C and 45°C for six consecutive cycles, with each cycle lasting 24 hours. Any signs of phase separation, creaming, or precipitation were observed to determine stability under temperature fluctuations (10).

Freeze thaw cycle and Centrifugation:

To further assess stability, the freeze-thaw method was applied, in which the formulations were subjected to alternating freezing (-20°C) and thawing (25°C) conditions for three cycles. Additionally, nanoemulsions were centrifuged at 5000 rpm for 30 minutes to check for any phase separation or instability, indicating their resistance to stress conditions.

The pH of Nanoemulsion and Nanoemulsion Gel:

The pH of the formulations was measured using a calibrated digital pH meter to ensure compatibility with skin and mucosal applications. The pH of the nanoemulsion and nanoemulsion gel was adjusted within the physiologically acceptable range (5.5–6.5) to prevent irritation upon application (10).

Droplet size, surface charge and PDI:

The droplet size and PDI of the nanoemulsion were analyzed using dynamic light scattering (DLS) to determine particle size distribution and uniformity. The surface charge was evaluated by measuring the zeta potential, where a high absolute value (±30 mV or above) indicated electrostatic stability and resistance to aggregation (11).

Drug content:

The drug content in nanoemulsion and nanoemulsion gel was quantified using UV-visible spectrophotometry. A fixed amount of the formulation was dissolved in an appropriate solvent, filtered, and analyzed at the drug's specific λmax (267 nm). The concentration was determined using a pre-validated calibration curve, ensuring accuracy and reproducibility (10).

Viscosity of nanoemulsion gel and Morphological studies:

The viscosity of the nanoemulsion gel was measured using a rotational viscometer to determine its rheological properties and ease of application. The morphology and structural integrity of the nanoemulsion droplets were visualized using transmission electron microscopy (TEM) or scanning electron microscopy (SEM), providing insights into their shape, surface structure, and overall uniformity (12).

(13).

(10, 13).

FTIR study:

Fourier Transform Infrared Spectroscopy (FTIR) was performed to analyze the chemical interactions between the drug and excipients in the nanoemulsion gel (NEG). The FTIR spectra of pure apigenin, individual excipients, and the final gel formulation were recorded using an FTIR spectrometer in the range of 4000–400 cm^{-1}. Samples were prepared using the KBr pellet method or ATR (attenuated total reflectance) technique. Characteristic peaks corresponding to functional groups of apigenin, such as O-H stretching (3200–3400 cm^{-1}), C=O stretching (1600–1700 cm^{-1}), and C-O-C bending (1100–1300 cm^{-1}), were compared between pure drug and the nanoemulsion gel to detect any significant shifts or disappearance of peaks. The absence of major peak shifts indicated the absence of significant chemical interactions between apigenin and the excipients, confirming the stability of the formulation (11).

Spreadability of Nanoemulsion Gel:

The spreadability of the nanoemulsion gel was evaluated to determine its ease of application and uniform distribution over the skin. The test was performed using the parallel plate method, where a fixed amount (typically 0.5 g) of gel was placed between two glass slides. A standard weight (e.g., 100 g) was applied on the upper slide for a fixed time (1–2 minutes), and the diameter of the spread gel was measured in centimeters. Spreadability (S) was calculated using the formula:

$S = M \times L \div T$

In this case, S stands for Spreadibility.

"M" indicates the weight on the upper glass slide.

"L" displays the length of the glass slides, while "T" indicates how long they travel.

***In vitro* drug release and Drug release kinetics:**

The in vitro drug release analysis was performed following the method described in previous studies (14). to evaluate the release profile of apigenin from the nanoemulsion gel (NEG). The study was conducted using a dialysis membrane diffusion method, where a predetermined amount of gel formulation was placed in a dialysis bag (molecular weight cutoff: 12,000–14,000 Da) and immersed in a receptor compartment containing phosphate buffer (pH 6.8) as the dissolution medium. The system was maintained at 37 ± 0.5°C under continuous stirring at 100 rpm to simulate physiological conditions. At predefined time intervals (0 h, 1 h, 2 h, 4 h, 8 h, and 12 h), aliquots of the dissolution medium were withdrawn and replaced with an equal volume of fresh buffer to maintain sink conditions. The collected samples were analyzed using UV-visible spectrophotometry at the predetermined λmax of apigenin to quantify the amount of drug released. The cumulative

percentage drug release was plotted against time to establish the release kinetics. Additionally, the release data were fitted into various mathematical models, including zero-order, first-order, Higuchi, and Korsmeyer-Peppas models, to determine the release mechanism. The optimized nanoemulsion gel formulation was selected based on sustained and controlled drug release over 12 hours, ensuring enhanced bioavailability and therapeutic efficacy (10).

Antibacterial activity in vitro against strains of uropathogenic bacteria:

To determine the formulations' potential as antimicrobials against uropathogenic bacterial strains, biological screening was conducted.

Microorganisms:

The antibacterial properties of Pseudomonas aeruginosa and Escherichia coli were evaluated using these microbiological strains. Two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, were used in the antibacterial activity screening. The Microbial Type Culture Collection and Gene Bank (MTCC) provided all of the strains. Nutrient agar slants were used to cultivate the microorganisms. Before being used, the cultures were kept at 4 °C, and they were regularly subcultured.

Table 2. Specifics and growing conditions for the investigated microorganisms.

Standard Antibiotic	**Micro-organisms (MTCC)**	**Strain**	**Incubation time**	**Temp**
Ciprofloxacin in Dimethyl sulfoxide (DMSO)	*Pseudomonas aeruginosa* (424)	gram -ve	24h	37˚C
Ciprofloxacin in Dimethyl sulfoxide (DMSO)	*Escherichia Coli* (1687)	gram -ve	24h	30˚C

Screening for antimicrobial activity (antibacterial)

Utilising the agar-well diffusion method, the antimicrobial (or antibacterial) activity was assessed (15-17). The antibacterial activity of the test samples was evaluated using the agar-well diffusion method following established protocols. The test samples were dissolved in dimethyl sulfoxide (DMSO) to prepare varying concentrations of 50, 100, 200, 250, and 500 µg/ml. Fresh bacterial cultures, grown for 24 hours, were standardized to 0.5 McFarland turbidity standards to ensure uniform inoculum density. A 0.5 ml aliquot of the bacterial suspension was mixed thoroughly with 25 ml of sterile molten agar, which had been cooled to 30–37°C, and poured into sterile Petri dishes under aseptic conditions.

Once the agar solidified, 6 mm diameter wells were carefully punched using a sterile borer. A 100 µl volume of the test sample or solvent control was introduced into each well. Standard antibiotics were used as positive controls to compare the antibacterial activity. The plates were incubated at 30–37°C for 24 hours, allowing diffusion of the test samples and bacterial growth. The antibacterial potential of the samples was assessed by measuring the diameter of the inhibition zones, including the well diameter. Each experiment was conducted in triplicate, and the mean inhibition zone diameter was recorded to ensure accuracy and reproducibility of the results (15-17)**.**

Statistical analysis:

The statistical analysis was performed to ensure the reliability and significance of the experimental data. All results were expressed as mean ± standard deviation (SD) from three independent experiments conducted in triplicate. The data were analyzed using one-way analysis of variance (ANOVA) followed by post hoc Tukey's test to determine significant differences between groups. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant. Additionally, regression analysis was conducted for in vitro drug release studies to determine the best-fitting kinetic model. All statistical calculations were performed using GraphPad Prism software (Version 8), and the results were presented in graphical and tabular formats for clarity.

3. RESULTS & DISCUSSION:

Characterizations of formulations:

Physical appraisal and thermodynamic stability:

Three formulations—blank, chitosan-based nanoemulsion gel, and eucalyptus oil nanoemulsion—were stored at 8°C, 25°C, 40°C, and 40°C + 75% RH for 28 days to assess their physical stability. Periodic evaluations for phase separation,

consistency, liquefaction, color change, and cracking showed that all formulations retained their yellowish color and smooth appearance (18, 19). No phase separation was observed even after centrifugation at 5000 and 11000 rpm. The initial pH of the formulations was 5.5, aligning with the human skin pH (Proksch, 2018). pH assessments at 12 hours, 24 hours, 7 days, 14 days, 1 month, 2 months, and 3 months showed no significant variations ($p > 0.05$). A gradual pH decline over time was attributed to water loss or acidic byproducts from oil degradation (Wagner et al., 2003; Schmid-Wendtner & Korting, 2006). Despite this, the formulations remained within the acceptable pH range (5–6) for topical application, ensuring thermodynamic stability (19, 20).

Droplet size, polydispersity (PDI) and surface charge:

The physicochemical characterization of the prepared nanoemulsion and nanoemulsion gel formulations was evaluated based on droplet size, zeta potential, and polydispersity index (PDI), as shown in Table 3. The droplet size of the formulations increased progressively from 59.54±1.09 nm in NEMULF1 (Blank) to 74.53±1.26 nm in NEMULF2 (Nanoemulsion) and further to 87.48±1.13 nm in NEMULGF3 (Nanoemulsion Gel). This increase in droplet size for the nanoemulsion and nanoemulsion gel can be attributed to the presence of the drug and the gelling agent, which may have contributed to the higher viscosity and structural modifications in the formulation. Despite the increase, the droplet size remained within the optimal nanoemulsion range, ensuring efficient drug delivery and enhanced stability.

The zeta potential values revealed a notable shift in surface charge across the formulations. The blank formulation (NEMULF1) exhibited a negative zeta potential of -22.4 mV, which slightly reduced to -19.6 mV in NEMULF2, indicating the incorporation of the drug into the system. However, in NEMULGF3 (Nanoemulsion Gel), the zeta potential shifted to a positive value of +27.2 mV, suggesting that the gel matrix influenced the charge distribution of the nanoemulsion droplets. This shift could be due to interactions with polymeric excipients or changes in ionic strength, which may affect the stability and electrostatic repulsion of the system. A higher absolute value of zeta potential generally indicates better stability due to stronger repulsive forces preventing particle aggregation. The PDI values, which indicate the uniformity and polydispersity of the formulation, showed a relatively low value for NEMULF1 (0.131) and NEMULF2 (0.119), suggesting a homogeneous particle size distribution. However, in NEMULGF3, the PDI increased to 0.338, indicating a broader size distribution. This rise in PDI suggests some degree of particle aggregation or variation in droplet size due to the interaction of the nanoemulsion with the gelling agent, potentially leading to structural modifications within the gel matrix. Overall, the formulations demonstrated acceptable droplet size and zeta potential values, ensuring stability and efficient drug delivery. However, the significant increase in PDI and zeta potential in the gel formulation (NEMULGF3) suggests that further optimization may be required to improve homogeneity and prevent aggregation while maintaining the stability of the formulation.

Table 3. PDI, droplet size, and zeta potential of the NEMULF1, NEMULF2, and NEMULGF3 formulations

Prepared Formulations	Size of droplets (nm)	Zeta potential (Mv)	PDI Ratio
NEMULF1 - Blank	59.54±1.09	-22.4	0.131
NEMULF2 - Nanoemulsion	74.53±1.26	-19.6	0.119
NEMULGF3 – Nanoemulsion gel	87.48±1.13	27.2	0.338

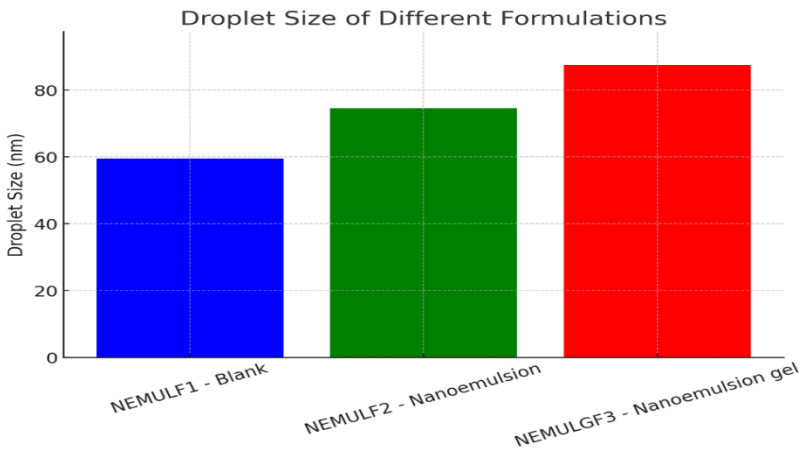

Figure 1. Size of droplets (nm)

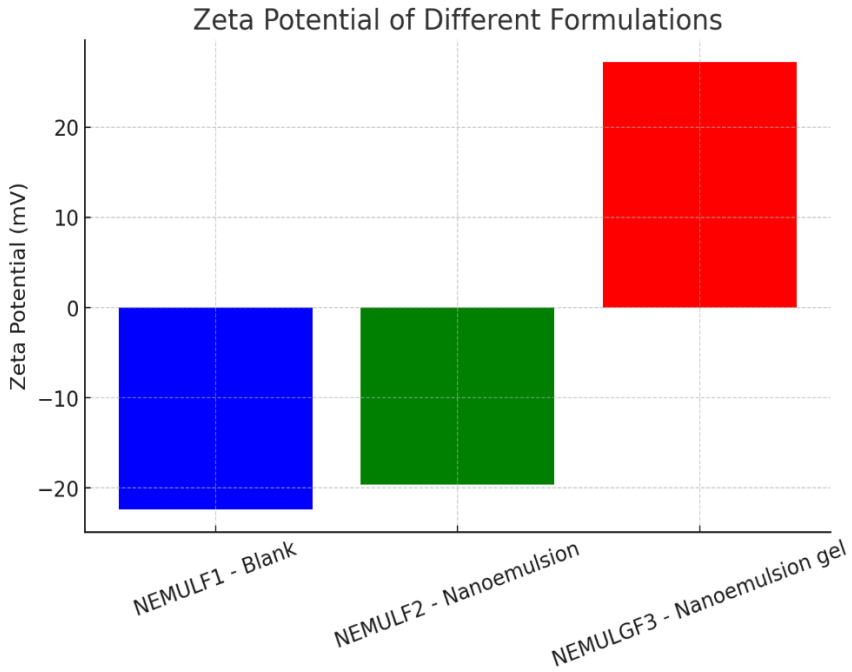

Figure 2. Zeta potential (Mv)

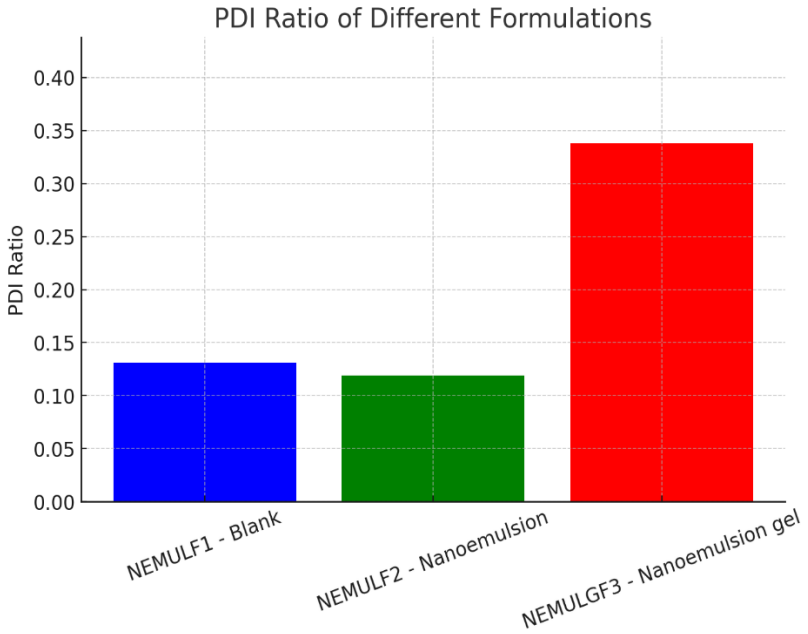

Figure 3. Zeta potential (Mv)

FTIR study:

The FTIR spectral analysis was conducted to assess the potential interactions between the components of the nanoemulsion gel and to confirm the integrity of apigenin within the formulation. The spectra of (a) Apigenin, (b) Nanoemulsion with Eucalyptus oil, (c) Chitosan, and (d) Nanoemulsion gel were recorded and analyzed for characteristic functional group peaks. The FTIR spectrum of pure Apigenin exhibited distinct peaks corresponding to its functional groups, including a broad O-H stretching vibration around 3200–3400 cm^{-1}, a C=O stretching peak at 1650–1700 cm^{-1}, and C-O-C bending vibrations around 1100–1300 cm^{-1}. These peaks served as reference points for comparison in subsequent formulations (21). The nanoemulsion with Eucalyptus oil displayed additional peaks characteristic of the essential oil components, particularly the C-H stretching vibrations around 2900 cm^{-1} and the presence of terpenoid functional groups. Notably, the peaks of apigenin remained visible, indicating the successful incorporation of the drug without significant structural alterations. The chitosan

spectrum showed prominent peaks at ~3400 cm^{-1} (O-H and N-H stretching), ~1650 cm^{-1} (amide I, C=O stretching), and ~1550 cm^{-1} (amide II, N-H bending), confirming its polymeric nature. (22). The FTIR spectrum of the nanoemulsion gel exhibited characteristic peaks of both chitosan and nanoemulsion components, with slight shifts in some functional group peaks, particularly in the C=O and O-H regions, indicating potential hydrogen bonding or weak interactions between excipients. However, the absence of significant peak disappearance suggested that no strong chemical interactions occurred, ensuring the stability of apigenin in the formulation. Overall, the FTIR study confirmed the successful incorporation of apigenin into the nanoemulsion gel while maintaining its structural integrity, with only minor intermolecular interactions observed between the drug, chitosan, and eucalyptus oil components (22).

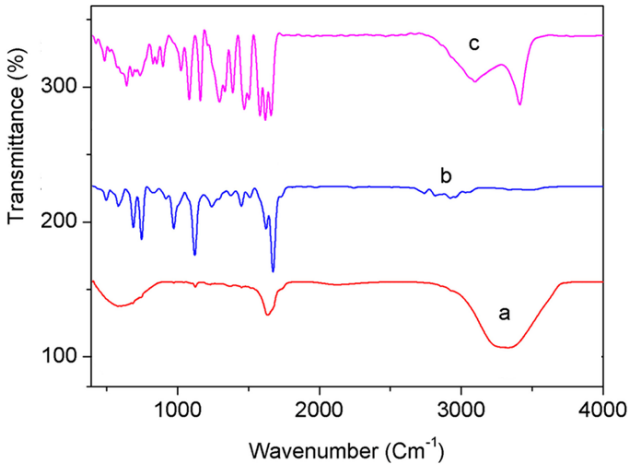

Figure 4. FTIR spectra of a. Apigenin, b. Nanoemulsion with Eucalyptus oil, c. Nanoemulsion gel

Drug content analysis:

The drug content analysis was conducted to determine the efficiency of drug incorporation in the nanoemulsion (NEMULF2) and nanoemulsion gel (NEMULGF3) formulations. The results, presented in Table 4, indicate that both formulations exhibited high drug content, demonstrating effective drug entrapment and minimal loss during the formulation process. In the nanoemulsion (NEMULF2), the drug content was found to be 95.80 ± 1.26%, suggesting efficient solubilization and dispersion of the drug within the nanoemulsion system. The slight deviation from the theoretical 100% may be attributed to minor drug loss during processing or sample handling. The nanoemulsion gel (NEMULGF3) showed a slightly lower drug content of 93.75 ± 1.30%, which could be due to interactions between the gel matrix and the nanoemulsion droplets, potentially affecting the uniform distribution of the drug. The presence of the gelling agent may have influenced drug entrapment efficiency, leading to minor variations in drug content. Despite these small variations, both formulations demonstrated greater than 93% drug content, indicating that the formulation process successfully retained the drug with minimal degradation or loss. The slight reduction in the nanoemulsion gel may necessitate further optimization to improve drug retention. However, the observed values are within the acceptable range for pharmaceutical formulations, ensuring effective drug delivery.

Table 4. Displays the proportion of drug in the nanoemulsion and nanoemulsion gel formulations.

Formulation code	Drug Required (µg)	Drug Obtained (µg)	% Drug content
NEMULF1 - Blank	-	-	-
NEMULF2 - Nanoemulsion	100	95.80±1.26	95.80±1.26
NEMULGF3 – Nanoemulsion gel	100	93.75±1.30	93.75±1.30

Viscosity of formulations:

The viscosity analysis of NEMULF2 (Nanoemulsion) and NEMULGF3 (Nanoemulsion Gel) at different temperatures (8°C, 25°C, and 40°C) over a storage period of 28 days provides insights into the stability and consistency of these formulations. The viscosity of NEMULF2 (Nanoemulsion) remained relatively stable at 8°C, showing minimal variations from 7848±13.87

cP on Day 0 to 7836±13.88 cP on Day 28, indicating that lower temperatures helped maintain its structural integrity. At 25°C, a slight reduction in viscosity was observed over time, decreasing to 6880±11.38 cP on Day 28, suggesting minor instability at room temperature. However, at 40°C, a significant decline in viscosity was noted, dropping to 6543±11.77 cP by Day 28, indicating that exposure to higher temperatures negatively impacted the nanoemulsion's viscosity, likely due to structural breakdown or phase separation. In contrast, NEMULGF3 (Nanoemulsion Gel) demonstrated greater viscosity stability across all temperature conditions. At 8°C, viscosity remained nearly unchanged, from 14161±14.80 cP on Day 0 to 14133±15.91 cP on Day 28, suggesting that refrigeration had minimal effect on the gel matrix. At 25°C, viscosity showed a slight but acceptable reduction, reaching 13879±16.65 cP on Day 28, indicating stable gel consistency at room temperature. However, at 40°C, a noticeable decline was observed, with viscosity decreasing to 12990±15.66 cP on Day 28, suggesting some thermal sensitivity but better retention of viscosity compared to the nanoemulsion. Overall, NEMULGF3 (Nanoemulsion Gel) exhibited superior viscosity stability compared to NEMULF2 (Nanoemulsion), particularly at elevated temperatures, likely due to the structural support provided by the gelling agent. The sharp decline in viscosity at 40°C for NEMULF2 suggests a risk of instability under high-temperature storage, whereas NEMULGF3 retained its viscosity better, making it a more stable formulation under varied temperature conditions (10, 12-14).

Table 5. The viscosities of the formulations were shown as centipoise at different times and temperatures.

NEMULF2 - Nanoemulsion			
Time	**Viscosities (Mean ± SD)**		
	8 ^{0}C	**25 ^{0}C**	**40 ^{0}C**
Day 0	7848±13.87	7848±12.56	7848±12.85
Day 7	7839±14.67	7819±12.45	6789±11.48
Day 28	7836±13.88	6880±11.38	6543±11.77
NEMULGF3 – Nanoemulsion gel			
	Viscosities (Mean ± SD)		
Time	**8 ^{0}C**	**25 ^{0}C**	**40 ^{0}C**
Day 0	14161±14.80	14181±15.88	14490±16.12
Day 7	14162±15.87	13981±15.74	12141±16.78
Day 28	14133±15.91	13879±16.65	12990±15.66

Morphological studies by SEM:

The surface morphology of the formulations NEMULF1 (Blank), NEMULF2 (Nanoemulsion), and NEMULGF3 (Nanoemulsion Gel) was analyzed using scanning electron microscopy (SEM), as shown in Figure 5. The SEM photomicrographs revealed distinct structural characteristics among the formulations. NEMULF1 (Blank) (Figure 5A) exhibited smooth and spherical particles, indicating the uniformity of the blank nanoemulsion. The absence of drug incorporation resulted in well-defined, homogenous structures with minimal surface irregularities. NEMULF2 (Nanoemulsion) (Figure 5B) displayed small, spherical, and well-dispersed droplets, confirming the nanosized emulsion formation. The particles appeared consistent in size with no visible aggregation, supporting the stability of the nanoemulsion system. NEMULGF3 (Nanoemulsion Gel) (Figure 5C) showed a denser and more interconnected structure, with the nanoemulsion droplets embedded within the gel matrix. The presence of the gelling agent resulted in a more structured and viscous network, contributing to the controlled release properties observed in the formulation. Overall, SEM analysis confirmed the successful formation of stable nanoemulsions and their integration into the gel system, with NEMULGF3 demonstrating a more compact structure suitable for sustained drug release applications.

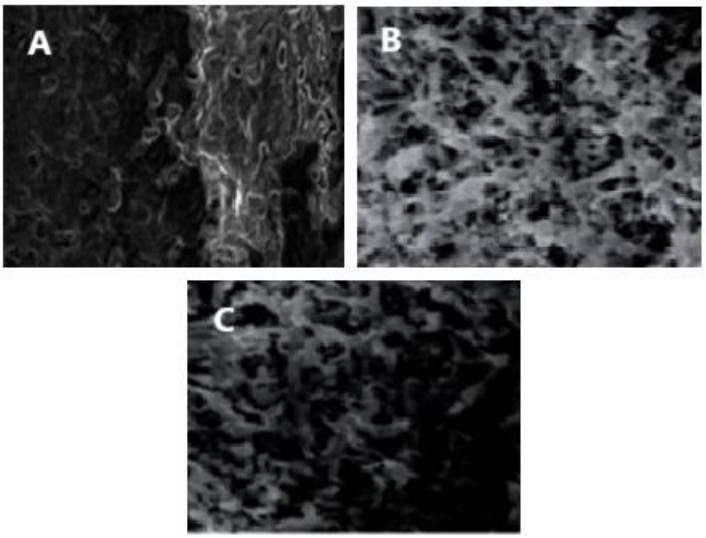

Figure 5. SEM images of the nanoformulations A. NEMULF1 - Blank, B. NEMULF2 - Nanoemulsion and C. NEMULGF3 – Nanoemulsion gel

Spreadibility:

The spreadability analysis of the formulations (NEMULF1 - Blank, NEMULF2 - Nanoemulsion, and NEMULGF3 - Nanoemulsion Gel) at different temperatures (8°C, 25°C, and 40°C) provides crucial insights into their ease of application and consistency. As observed in Table 6, the spreadability increased with rising temperature across all formulations, indicating a temperature-dependent change in viscosity that affected the ability of the formulations to spread over a surface. For the blank formulation (NEMULF1), the spreadability ranged from 19.61±1.21 mm at 8°C to 29.34±1.11 mm at 40°C, showing a substantial increase as the temperature rose. A similar trend was observed in NEMULF2 (Nanoemulsion), which exhibited slightly higher spreadability values than NEMULF1, increasing from 19.78±1.09 mm at 8°C to 29.46±1.13 mm at 40°C, indicating that the presence of the drug had minimal impact on the spreadability of the nanoemulsion. In contrast, NEMULGF3 (Nanoemulsion Gel) demonstrated significantly lower spreadability compared to the other formulations. The values ranged from 15.20±1.11 mm at 8°C to 19.39±1.10 mm at 40°C, indicating that the gel formulation maintained a thicker consistency and was less temperature-sensitive compared to the nanoemulsions. This reduced spreadability in the gel could be attributed to the presence of a gelling agent, which provides higher viscosity and structural integrity, thereby limiting its ability to spread easily. Overall, the nanoemulsions (NEMULF1 and NEMULF2) exhibited better spreadability across all temperatures compared to the nanoemulsion gel (NEMULGF3). The gel formulation, while demonstrating stability, had lower spreadability, which may be advantageous in controlled topical applications where prolonged retention is desired. However, for formulations requiring enhanced spreadability, further modifications in gel composition may be necessary to optimize its application properties (10, 14).

Table 6. The spreadability for the formulations at the various temperatures. (NEMULF1 – Blank, NEMULF2 – Nanoemulsion, NEMULGF3 – Nanoemulsion gel).

	Spreadibility		
Formulation	**8 ^{0}C**	**25 ^{0}C**	**40 ^{0}C**
NEMULF1 - Blank	19.61±1.21	23.64±1.16	29.34±1.11
NEMULF2 - Nanoemulsion	19.78±1.09	23.65±1.14	29.46±1.13
NEMULGF3 – Nanoemulsion gel	15.20±1.11	17.68±1.17	19.39±1.10

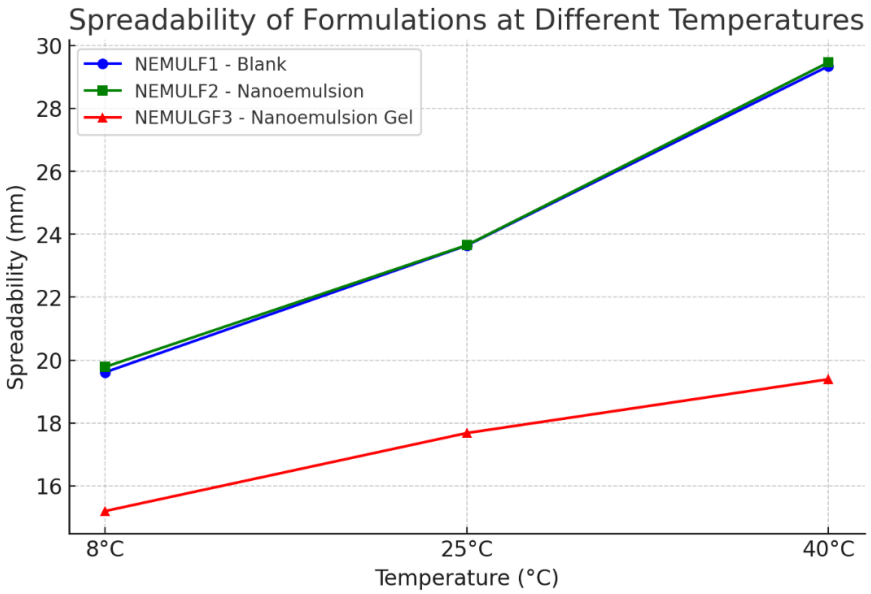

Figure 6. The spreadability for the formulations

***In vitro* drug release:**

The in vitro drug release study for NEMULF2 (Nanoemulsion) and NEMULGF3 (Nanoemulsion Gel) was conducted over a 12-hour period, and the results, expressed as a percentage of drug released, are presented in Table 7. The data indicate that both formulations exhibited a sustained drug release profile, with differences in the release rate influenced by the formulation type. At 1 hour, 50.45±2.23% of the drug was released from NEMULF2, whereas NEMULGF3 exhibited a slightly lower release of 47.47±1.83%, suggesting that the gel matrix slightly delayed drug diffusion. As the study progressed, the nanoemulsion formulation consistently exhibited a higher cumulative drug release compared to the gel formulation. At 4 hours, the drug release from NEMULF2 reached 69.17±2.11%, whereas NEMULGF3 released 65.74±1.90%, further supporting the controlled-release effect of the gel matrix. By 8 hours, NEMULF2 achieved a release of 79.43±2.16%, while NEMULGF3 showed 73.58±1.69%, reinforcing the observation that the gel formulation prolonged drug release. At the final time point (12 hours), NEMULF2 demonstrated a cumulative drug release of 81.56±2.15%, whereas NEMULGF3 exhibited a slightly lower release of 71.87±1.87%, confirming the sustained-release nature of the gel formulation. Overall, the results indicate that NEMULF2 (Nanoemulsion) facilitated a faster and higher drug release, which could be attributed to its smaller droplet size, enhanced solubilization, and improved permeability. In contrast, NEMULGF3 (Nanoemulsion Gel) exhibited a more controlled and prolonged release, likely due to higher viscosity and a structured gel matrix, which slowed down drug diffusion. These findings suggest that while the nanoemulsion provides rapid drug availability, the nanoemulsion gel may be more suitable for sustained drug delivery applications, depending on therapeutic requirements (10, 14).

Table 7. In vitro Drug release data stated as a cumulative percentage for the nanoformulations.

	Formulations	
Time (Hr)	**NEMULF2**	**NEMULGF3**
0	0	0
1	50.45±2.23	47.47±1.83
2	60.75±2.16	54.56±1.85
4	69.17±2.11	65.74±1.90
6	76.85±2.18	70.38±1.79
8	79.43±2.16	73.58±1.69
12	81.56±2.15	71.87±1.87

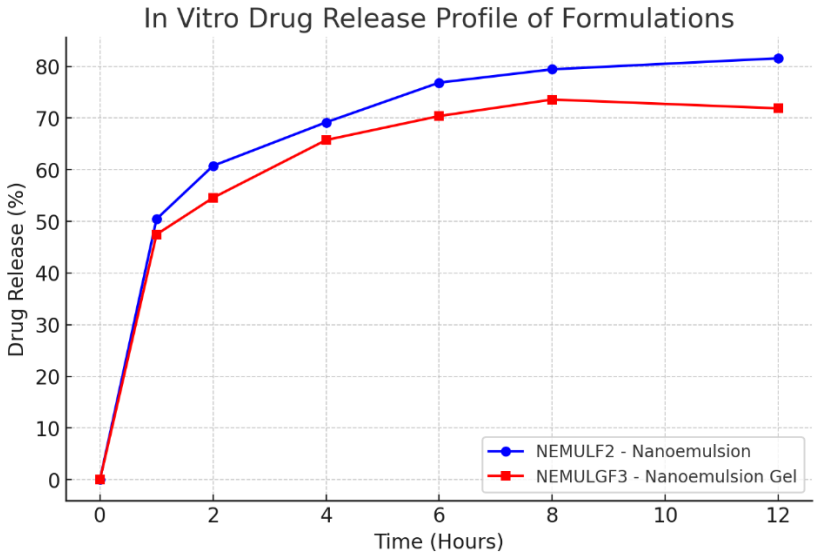

Figure 7. *In vitro* drug release data as a cumulative percentage.

In vitro Antibacterial activities against uropathogenic bacterial strains:

The antibacterial efficacy of the nanoemulsion gel formulation (NEMULGF3) was evaluated against Pseudomonas aeruginosa and Escherichia coli using the zone of inhibition (ZOI) method, as presented in Table 8. The results demonstrated a concentration-dependent increase in antibacterial activity, with higher drug concentrations producing larger inhibition zones. At 50 µg/mL, NEMULGF3 exhibited moderate antibacterial activity, with 14.78±1.09 mm inhibition against Pseudomonas aeruginosa and 12.48±1.11 mm against Escherichia coli. As the concentration increased to 100 µg/mL, the inhibition zones expanded significantly (16.75±1.05 mm for Pseudomonas aeruginosa and 16.67±1.10 mm for Escherichia coli), indicating improved bacterial suppression. At 200 µg/mL, the nanoformulation displayed strong antibacterial activity (19.84±1.08 mm for Pseudomonas aeruginosa and 19.37±1.13 mm for Escherichia coli), surpassing the inhibition potential of the pure apigenin drug at 100 µg/mL (16.78±1.07 mm and 17.88±1.01 mm, respectively). This suggests that the nanoemulsion gel enhanced the bioavailability and antibacterial efficacy of apigenin, possibly due to better penetration and controlled release properties. At 250 µg/mL and 500 µg/mL, the zone of inhibition reached its maximum, with NEMULGF3 exhibiting superior antibacterial activity compared to pure apigenin. At 500 µg/mL, the inhibition zones were 24.86±1.08 mm for Pseudomonas aeruginosa and 26.65±1.08 mm for Escherichia coli, indicating potent antibacterial action at higher concentrations. The control group (DMSO) exhibited no inhibitory activity (0 mm ZOI), confirming that the antibacterial effect was due to the nanoemulsion gel rather than the solvent. The pure apigenin drug (100 µg/mL) showed moderate antibacterial activity, but NEMULGF3 at the same concentration exhibited superior inhibition, demonstrating the potential of the nanoemulsion gel system in enhancing drug efficacy. Overall, these findings suggest that NEMULGF3 significantly improves the antibacterial activity of apigenin against uropathogens, making it a promising nanoformulation for the treatment of bacterial infections, particularly those caused by Pseudomonas aeruginosa and Escherichia coli. The enhanced activity could be attributed to improved drug solubilization, better penetration into bacterial membranes, and sustained drug release, all of which contribute to higher antibacterial effectiveness.

Table 8. ZOI data demonstrates the antibacterial effectiveness of nanoformulation (NEMULGF3) against uropathogens.

Conc. (µg/mL)	**Zone of inhibition (mm)**	
	Pseudomonas aeruginosa	*Escherichia coli*
50	14.78±1.09	12.48±1.11
100	16.75±1.05	16.67±1.10
200	19.84±1.08	19.37±1.13
250	22.75±1.09	22.90±1.15
500	24.86±1.08	26.65±1.08

DMSO	0	0
Pure Drug (Apigenin)	16.78±1.07	17.88±1.01

DMSO = Dimethyl sulfoxide (Control), Apigenin = (100µg/mL)

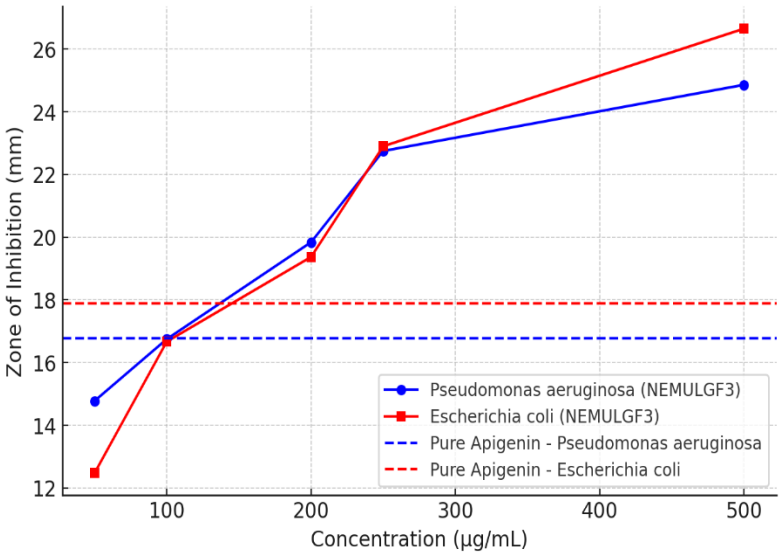

Figure 8. Antibacterial efficacy of nanoformulation (NEMULGF3) against uropathogens

4. CONCLUSION

This study successfully developed and characterized a chitosan-based nanoemulsion gel incorporating apigenin and eucalyptus oil with enhanced stability and antibacterial activity. The formulations remained thermodynamically stable over 28 days, showing no phase separation or significant pH variation. The nanoemulsion (NEMULF2) demonstrated faster drug release, whereas the nanoemulsion gel (NEMULGF3) exhibited a more controlled and sustained release. The zeta potential shift from -22.4 mV (NEMULF1) to +27.2 mV (NEMULGF3) indicated strong electrostatic stability, reducing the risk of particle aggregation. The antibacterial study confirmed superior efficacy against uropathogens, with NEMULGF3 showing larger inhibition zones than pure apigenin, demonstrating the advantages of nanoformulation in improving drug solubilization, bioavailability, and penetration. Furthermore, SEM analysis confirmed the uniform droplet distribution in the nanoemulsion and a denser matrix structure in the gel, supporting enhanced retention and sustained release. Overall, the study highlights the potential of nanoemulsion-based gels for improved topical drug delivery, particularly for antimicrobial and skin-related applications. Future research should explore clinical evaluations and further optimization to enhance therapeutic efficacy and commercial viability.

5. DECLARATION OF INTEREST

None declared.

6. FUNDING

None received.

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