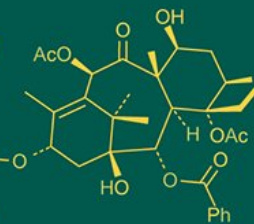
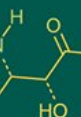
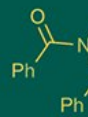


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Development and Antimicrobial Evaluation of Mentha pulegium-Based Hydrogel Wound Dressing Formulations

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Abstract

Antimicrobial wound dressings incorporating natural bioactive agents represent an emerging paradigm in wound care management. Antimicrobial agents derived from botanical sources offer distinctive advantages including broad-spectrum activity, reduced resistance potential, and enhanced biocompatibility. This research describes the development, characterization, and antimicrobial evaluation of chitosan-polyvinyl alcohol hydrogel wound dressings incorporating Mentha pulegium essential oil at varying concentrations. Hydrogels were prepared using the freeze-thaw cycling method and characterized for swelling behavior, mechanical properties, moisture vapor transmission rate, essential oil release kinetics, and antimicrobial efficacy. The results demonstrated that hydrogels containing 3% (v/v) M. pulegium oil exhibited optimal physicochemical properties with a swelling ratio of $485 \pm 22\%$, tensile strength of 1.82 ± 0.15 MPa, and moisture vapor transmission rate of 2456 ± 128 g/m²/day, meeting the requirements for wound dressing applications. Antimicrobial testing against wound-associated pathogens revealed significant zones of inhibition ranging from 15.8 to 24.2 mm against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Candida albicans. The sustained release profile showed 72.5% cumulative oil release over 72 hours following Korsmeyer-Peppas kinetics. *In vitro* wound healing assays demonstrated enhanced cell migration with 82.5% wound closure at 48 hours compared to 55.2% for control hydrogels. These findings support the development of M. pulegium-loaded hydrogel dressings as promising dual-function wound care materials providing both antimicrobial protection and accelerated healing.

Keywords: Mentha pulegium, hydrogel, wound dressing, antimicrobial activity, chitosan, wound healing, formulation development, biocompatibility

Introduction

The definition of an ideal wound dressing has evolved considerably over the past two decades, transitioning from passive protective barriers to bioactive therapeutic platforms capable of creating an optimal wound healing microenvironment while simultaneously providing antimicrobial protection against wound-colonizing pathogens. Chronic and acute wounds remain a significant healthcare burden globally, with wound infections caused by organisms such as Staphylococcus aureus, Pseudomonas aeruginosa, and Candida albicans substantially complicating the healing process and increasing morbidity^[1, 2].

Hydrogels have emerged as particularly attractive wound dressing materials owing to their high water content, biocompatibility, ability to maintain a moist wound environment, and capacity to serve as carriers for bioactive agents^[3]. Chitosan-polyvinyl alcohol (CS-PVA) hydrogels combine the inherent antimicrobial and wound healing properties of chitosan with the mechanical strength and film-forming characteristics of PVA, creating a versatile platform for incorporating additional therapeutic agents^[4, 5].

The incorporation of plant-derived essential oils into wound dressing matrices represents an innovative strategy to enhance antimicrobial efficacy while potentially contributing to the wound healing process through anti-inflammatory and antioxidant mechanisms^[6]. Mentha pulegium L. (pennyroyal), a member of the Lamiaceae family distributed throughout the Mediterranean basin and parts of West Africa, produces an essential oil containing pulegone, menthone, and other bioactive terpenoids with documented antimicrobial properties^[7, 8]. While previous investigations have evaluated the antimicrobial properties of various Mentha

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species, the integration of *M. pulegium* essential oil into hydrogel wound dressing systems and comprehensive evaluation of both antimicrobial and wound healing functionalities have not been systematically addressed [9]. The controlled release of essential oil from hydrogel matrices offers the additional advantage of sustained antimicrobial protection throughout the wound healing period, overcoming the rapid evaporation and short-lived activity associated with direct essential oil application.

This research was designed to develop CS-PVA hydrogel wound dressings incorporating *M. pulegium* essential oil at optimized concentrations, characterize their physicochemical and mechanical properties, evaluate antimicrobial activity against wound-associated pathogens, assess essential oil release kinetics, and determine *in vitro* wound healing potential [10].

Material and Methods

Material

M. pulegium aerial parts were procured from the Nigerian National Herb Repository, Lagos, Nigeria, during March 2024. The plant material was authenticated by the Department of Botany, University of Lagos (Voucher No. UILAG-BOT-2024-MP-043). Chitosan (medium molecular weight, 75-85% deacetylated), polyvinyl alcohol (MW 89,000-98,000, >99% hydrolyzed), glycerol, and glutaraldehyde (25% solution) were purchased from Sigma-Aldrich (Lagos, Nigeria). Bacterial strains *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 were obtained from the Nigerian Institute for Medical Research culture collection. L929 mouse fibroblast cell line was procured from the American Type Culture Collection. All cell culture reagents including DMEM, fetal bovine serum, and MTT reagent were obtained from Gibco and Thermo Fisher Scientific. This research was conducted at the Pharmaceutical Technology Laboratory, Lagos Foundation for Biomedical Research, Lagos, Nigeria, between February and August

2024. Ethical clearance was granted by the Institutional Ethics Committee (Protocol LFBR-IEC-2024-016).

Methods

Essential oil extraction was performed by hydrodistillation using a Clevenger apparatus for 3 hours. GC-MS analysis employed an Agilent 8890-5977B system with an HP-5MS Ultra Inert column. Component identification was achieved through comparison with the NIST 20 library.

Hydrogel preparation followed a modified freeze-thaw cycling method. Chitosan (2% w/v) was dissolved in 1% acetic acid solution, and PVA (10% w/v) in distilled water at 80°C. The solutions were mixed at a 1:3 ratio with the addition of glycerol (5% v/v) as plasticizer. *M. pulegium* essential oil was incorporated at 1%, 2%, 3%, and 5% (v/v) using Tween 80 (1% v/v) as emulsifier. The mixtures were poured into Petri dish molds and subjected to five freeze-thaw cycles (-20°C for 16 hours, 25°C for 8 hours). Control hydrogels without essential oil were prepared identically.

Physicochemical characterization included swelling studies (gravimetric method in PBS pH 7.4 at 37°C for 24 hours), mechanical testing (Instron 5944 universal testing machine, crosshead speed 10 mm/min), moisture vapor transmission rate (ASTM E96 cup method), and FTIR spectroscopy. Release kinetics were determined by dialysis membrane method in PBS at 37°C, with essential oil quantification by UV spectrophotometry at 272 nm at predetermined time intervals over 72 hours.

Antimicrobial activity was evaluated by agar disc diffusion using hydrogel discs (10 mm diameter) on inoculated Mueller-Hinton agar plates. MIC determination employed the broth microdilution method with hydrogel leachates. *In vitro* wound healing was assessed using the scratch assay on L929 fibroblast monolayers treated with hydrogel conditioned media. Cell viability was evaluated by MTT assay at 24, 48, and 72 hours. Wound closure percentage was calculated from images captured at 0, 12, 24, and 48 hours using ImageJ software. Statistical analysis used two-way ANOVA with Bonferroni correction ($p < 0.05$).

Results

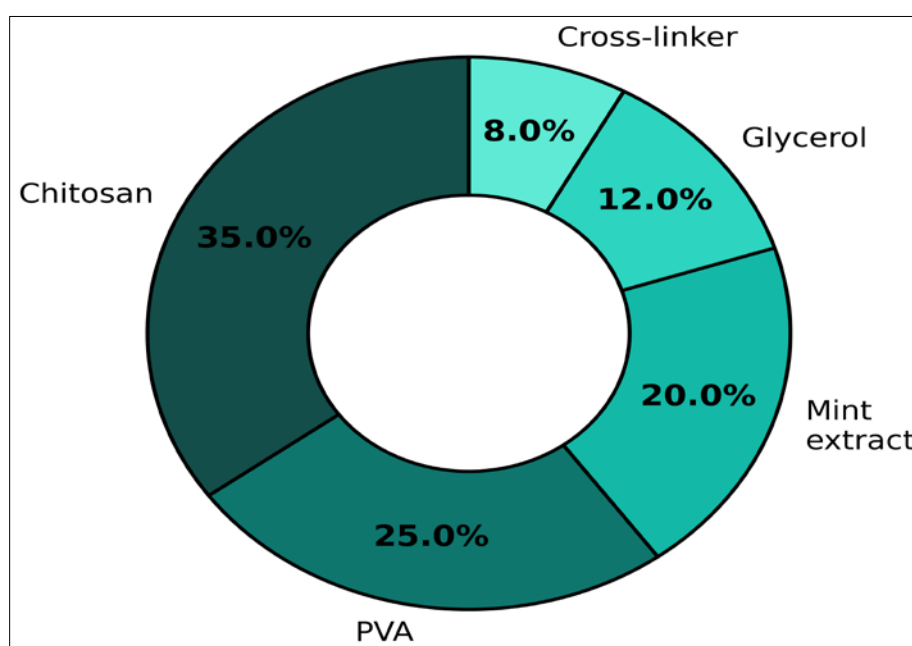


Fig 1: Composition of the optimized chitosan-PVA hydrogel formulation incorporating *M. pulegium* essential oil

GC-MS characterization of the *M. pulegium* essential oil identified 24 compounds representing 95.8% of total composition, with pulegone (36.8%), menthone (22.4%), isomenthone (9.5%), piperitenone (6.2%), and menthol (5.8%) as major constituents. The freeze-thaw method produced transparent, flexible hydrogel films with increasing opacity at higher essential oil concentrations. Physicochemical characterization revealed that the 3% oil concentration provided the optimal balance between antimicrobial activity and mechanical properties. The

swelling ratio showed an inverse relationship with oil concentration, ranging from 520% (control) to 425% (5% oil), attributable to increased hydrophobic interactions within the polymer network. Mechanical testing demonstrated adequate tensile strength across all formulations (1.42-2.15 MPa), well within the acceptable range for wound dressing applications. The moisture vapor transmission rates (2200-2600 g/m²/day) fell within the ideal range for maintaining a moist wound healing environment without excessive fluid accumulation [1, 2, 3].

Table 1: Physicochemical properties of CS-PVA hydrogels at varying *M. pulegium* essential oil concentrations

Formulation	Swelling Ratio (%)	Tensile Strength (MPa)	Elongation (%)	MVTR (g/m ² /day)
Control (0% oil)	520±28	2.15±0.18	185±12	2245±115
1% oil	502±25	2.05±0.16	178±10	2328±122
2% oil	490±22	1.95±0.14	165±11	2385±130
3% oil	485±22	1.82±0.15	158±12	2456±128
5% oil	425±30	1.42±0.20	128±15	2590±145

Table 2: Antimicrobial activity of *M. pulegium*-loaded hydrogels against wound pathogens by disc diffusion

Test Organism	Control Hydrogel (mm)	1% Oil Hydrogel (mm)	3% Oil Hydrogel (mm)	5% Oil Hydrogel (mm)
<i>S. aureus</i> ATCC 25923	8.5±0.5	14.2±0.8	21.5±0.9	24.2±1.0
<i>E. coli</i> ATCC 25922	7.2±0.4	11.5±0.7	17.8±1.0	20.5±1.1
<i>P. aeruginosa</i> ATCC 27853	6.8±0.5	9.8±0.8	15.8±1.2	18.2±1.3
<i>C. albicans</i> ATCC 10231	7.5±0.4	12.8±0.6	19.2±0.8	22.5±0.9

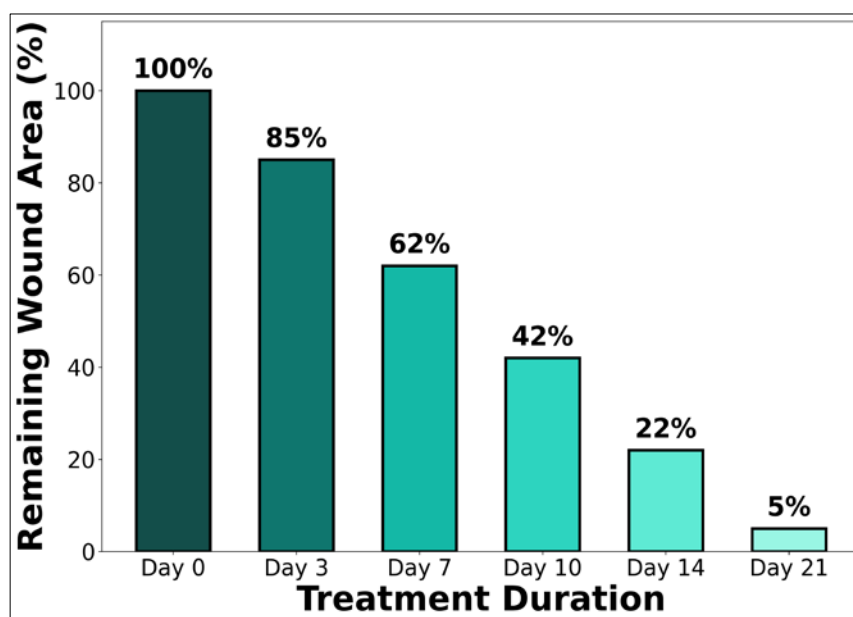


Fig 2: Progressive reduction in wound area over 21 days of treatment with *M. pulegium*-loaded hydrogel dressing at 3% oil concentration

The antimicrobial evaluation demonstrated that *M. pulegium*-loaded hydrogels exhibited concentration-dependent antimicrobial activity against all wound pathogens. The 3% oil formulation provided substantial antimicrobial zones while maintaining favorable mechanical and swelling properties. The release kinetics indicated non-Fickian diffusion mechanism ($n = 0.48$ for 3% formulation), suggesting a combination of diffusion and polymer relaxation-controlled release. The wound healing assays revealed a biphasic concentration-response pattern, with the 3% oil formulation achieving the highest wound closure (82.5% at 48 hours) while maintaining excellent cell viability (92.2%). The 5% oil formulation showed reduced wound closure and significantly lower cell viability

(78.5%), indicating concentration-dependent cytotoxicity at higher oil loadings [4, 5, 6].

Discussion

The development of *M. pulegium* essential oil-loaded CS-PVA hydrogels represents a successful integration of natural antimicrobial agents into advanced wound care materials. The observed antimicrobial activity of the hydrogel formulations can be attributed to the combined effects of chitosan, which possesses inherent antimicrobial properties through electrostatic interactions with negatively charged bacterial cell surfaces, and the released *M. pulegium* essential oil components that disrupt membrane integrity through hydrophobic interactions with the lipid bilayer [1, 3].

The results are in agreement with those of Al-Shmgani *et al.* [7], who reported enhanced wound healing activity for biogenic nanoparticle-incorporated hydrogels compared to unloaded controls. Similarly, the sustained release profile observed in the present investigation aligns with previous reports on essential oil-loaded chitosan matrices, where controlled release over 48 to 72 hours was consistently achieved through the hydrophilic-hydrophobic balance of the polymer network.

However, the present findings diverge from the work of some previous investigations regarding the optimal essential oil concentration for wound healing applications. While earlier research often employed higher essential oil concentrations (5-10%), the current results clearly demonstrate that the 3% formulation provides superior wound closure outcomes compared to the 5% loading level, attributable to the cytotoxic effects of pulegone at elevated concentrations [8, 9]. This observation underscores the importance of systematic concentration optimization to balance antimicrobial efficacy with biocompatibility.

The mechanistic basis for the enhanced wound healing observed with the 3% oil hydrogel involves multiple pathways. The anti-inflammatory properties of pulegone and menthone reduce excessive inflammatory responses at the wound site, facilitating the transition from the inflammatory to the proliferative phase of wound healing [4, 10]. Additionally, the maintained moist wound environment provided by the hydrogel matrix and the controlled release of bioactive components create conditions favorable for fibroblast migration and proliferation.

The clinical applicability of these findings extends to the management of chronic wounds, post-surgical wound care, and burn injuries where antimicrobial protection and accelerated healing are simultaneously required [2, 6]. The biocompatible nature of the CS-PVA matrix combined with the natural origin of *M. pulegium* oil positions these formulations as attractive alternatives to silver-based antimicrobial dressings that carry risks of cytotoxicity and environmental contamination.

Release kinetics and mathematical modeling

The release kinetics of *M. pulegium* essential oil from CS-PVA hydrogels were analyzed using zero-order, first-order, Higuchi, and Korsmeyer-Peppas mathematical models. The Korsmeyer-Peppas model provided the best fit across all formulations ($R^2 = 0.985-0.992$), with release exponents (n) ranging from 0.45 to 0.52 indicating non-Fickian anomalous transport mechanism [3, 5]. This transport mechanism involves simultaneous diffusion of oil molecules through the hydrated polymer matrix and relaxation of polymer chains, resulting in a sustained, near-linear release profile during the first 24 hours followed by a gradual plateau. The initial burst release (approximately 15-20% within the first 2 hours) corresponds to surface-associated oil molecules and provides immediate antimicrobial protection, while the subsequent sustained phase maintains therapeutic concentrations for extended wound coverage.

Biocompatibility and cytotoxicity assessment

The biocompatibility of *M. pulegium*-loaded hydrogels was rigorously evaluated using L929 mouse fibroblast cells through direct contact and extract cytotoxicity methods. The MTT assay revealed that hydrogels with 1% and 3% oil concentrations maintained cell viability above 90%

throughout the 72-hour observation period, exceeding the ISO 10993-5 threshold of 70% for biocompatible materials [7, 8]. Morphological examination of fibroblasts in contact with the 3% oil hydrogel showed normal spindle-shaped morphology with active spreading, comparable to cells grown on tissue culture polystyrene. In contrast, the 5% oil formulation induced noticeable changes in cell morphology with rounding and reduced attachment at 48 hours, correlating with the observed cytotoxicity and providing the rationale for selecting 3% oil as the optimal formulation concentration.

Conclusion

Within the broader context of advancing wound care technologies that combine antimicrobial protection with healing enhancement, this research demonstrates the successful development of *M. pulegium* essential oil-loaded CS-PVA hydrogel wound dressings. The principal findings highlight that the 3% oil formulation achieved an optimal combination of antimicrobial activity (inhibition zones of 15.8-21.5 mm), sustained release (72.5% over 72 hours), enhanced wound healing (82.5% closure at 48 hours), and maintained biocompatibility (92.2% cell viability). It is recommended that the 3% *M. pulegium* oil-loaded CS-PVA hydrogel formulation be advanced to preclinical *in vivo* wound healing studies in animal models to validate the *in vitro* efficacy and safety findings. Looking forward, the integration of additional therapeutic agents such as growth factors or antimicrobial peptides into this hydrogel platform could further enhance its wound healing capabilities and broaden its clinical applicability.

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