STANDARDIZED *Eucalyptus camaldulensis (Myrtaceae*) LEAF DERIVED TOPICAL PHYTOPHARMACEUTICAL GEL FOR MICROBIAL BIOFILM DISRUPTION AND INFECTION CONTROL IN DIABETIC FOOT ULCER

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ABSTRACT

Diabetic foot ulcers (DFUs) are chronic infections frequently associated with biofilm-forming bacterial pathogens that delay wound healing and complicate therapeutic management. The present study aimed to formulate and evaluate a topical gel containing the ethanolic extract of ***Eucalyptus camaldulensis*** and to investigate its antibacterial and antibiofilm potential against DFU-associated pathogens. Three gel formulations containing 1%, 2%, and 3% extract were prepared using Carbopol 934 as the gelling agent and evaluated for physicochemical properties including appearance, pH, spreadability, and viscosity. Antibacterial activity was assessed using the agar well diffusion method against the clinical isolate of ***Staphylococcus aureus*** obtained from diabetic foot ulcer samples. The antibiofilm potential of the formulations was determined using the crystal violet microtiter plate assay. All gel formulations exhibited satisfactory physicochemical properties with pH values ranging from 6.0 to 6.3, indicating suitability for topical application. The antibacterial assay demonstrated significant inhibitory activity of the formulations, particularly the 1% gel, while placebo and solvent controls showed no inhibition. The antibiofilm study revealed notable inhibition of biofilm formation, with the 3% gel formulation exhibiting the highest inhibition (82.81%). In comparison, standard drugs Linezolid and Silver sulfadiazine showed almost complete biofilm inhibition (99.9%). The results suggest

that the formulated gel possesses promising antibacterial and antibiofilm properties and may serve as a potential natural therapeutic agent for the management of diabetic foot ulcer infections.

Keywords: Diabetic foot ulcer, *Eucalyptus camaldulensis*, antibacterial activity, antibiofilm activity, gel formulation, *Staphylococcus aureus*

INTRODUCTION

Diabetic foot ulcers represent one of the most serious complications associated with diabetes mellitus and are a major cause of morbidity, hospitalization, and lower limb amputations worldwide[1,2,3]. Chronic wound infections in diabetic patients are frequently associated with microbial colonization and biofilm formation, which significantly impair wound healing and reduce the efficacy of conventional antimicrobial therapy[4,5]. Among the various pathogens implicated in diabetic foot infections, ***Staphylococcus aureus*** is one of the most predominant organisms due to its strong capacity for adhesion, colonization, and biofilm development [4,6].

Biofilm formation enables bacteria to survive in hostile environments by producing an extracellular polymeric matrix that protects them from antibiotics and host immune responses[4,5,6]. Consequently, the management of biofilm-associated infections requires alternative therapeutic strategies capable of inhibiting microbial adhesion and disrupting biofilm architecture.

Medicinal plants have gained increasing attention as potential sources of antimicrobial agents due to their diverse phytochemical constituents and comparatively lower risk of resistance development[7]. Among these, ***Eucalyptus camaldulensis*** has been widely reported for its antimicrobial, anti-inflammatory, and antioxidant properties[8,9,10]. The plant contains several bioactive compounds including phenolics, flavonoids, and terpenoids that contribute to its biological activities[7,8].

Topical gel formulations represent an effective drug delivery system for wound management because they provide sustained release, improved patient compliance, and enhanced local therapeutic action[11]. Therefore, the present study aimed to formulate a topical gel containing the ethanolic extract of ***Eucalyptus camaldulensis*** and evaluate its physicochemical properties, antibacterial activity, and antibiofilm potential against DFU-associated pathogens. Herbal hydrogels were selected over other conventional formulations due to their superior biocompatibility, enhanced patient compliance, and effective drug delivery characteristics. The

high water content of hydrogels provides a soothing and cooling effect, making them particularly suitable for topical applications. Additionally, hydrogels facilitate controlled and sustained release of herbal constituents, improving therapeutic efficacy. Their non-greasy nature, ease of application, and ability to enhance skin penetration further contribute to their preference. Moreover, the hydrogel system helps in maintaining the stability of herbal active compounds, thereby ensuring better overall performance compared to other formulations

MATERIAL AND METHOD

I . FORMULATION DEVELOPMENT OF EEEUC

The hydrogel formulation was prepared according to the standard procedure with slight modifications. Hydrogel formulation of EEEUC was developed as per following procedure

Ingredients	**Formulation I 1%**	**Formulation II 2%**	**Formulation III 3%**
EEEUC(1:9) (Ethanol:Water)	1 mg	2mg	3mg
Carbopol 2%	0.15gm	0.15gm	0.15gm
Triethanolamine	2ml	2ml	2ml
Glycerine	2ml	2ml	2ml
Methyl paraben	2ml	2ml	2ml
Distilled Water	qs	qs	qs

Table.no:1-Required Ingredients in the formulation of EEEUCG

Procedure

Disperse Carbopol first: Sprinkle 0.15 g Carbopol slowly into 4-5 mL distilled water while stirring vigorously (use magnetic stirrer) to form lump-free slurry. Let hydrate 30-60 min .Neutralize with TEA: Add triethanolamine drop wise (~0.5 mL) to Carbopol slurry with continuous stirring until clear gel forms (pH 6.5-7.0).

Avoid excess to prevent stickiness. Add extract. Incorporate 2 mL ***Eucalyptus Camaldulensis*** extract (1:9 ethanol:water) slowly to gel while stirring gently to maintain homogeneity .Mix glycerine. Add 2 mL glycerine; stir 5-10 min for humectant incorporation. Add methyl paraben last.

Dissolve 0.2 g methyl paraben in minimal warm water (~0.5 mL, <60°C); mix into final gel. qs. with distilled water to 10 g. Homogenize. Stir 15 min total; de-aerate under vacuum if available. Transfer to sterile jar**.**

II. EVALUATION OF EEEUC:

Evaluation of the formulated EEUCG hydrogel was carried out in accordance with pharmacopoeial standards, and the results are shown below."

The formulated Hydrogel was evaluated for the following parameters.

Appearance:

Observe clarity, homogeneity, uniformity, absence of lumps, phase separation, or cracks under normal light.

Color:

Note the colour of the **EEEUCG** gel formulations 1-3 ensuring consistency with formulation standards.

Odor:

Evaluate smell (e.g., characteristic, mild, or odorless) without heating, avoiding inhalation of volatile substances.

pH measurement:

The pH of the hydro gels was measured with the help of digital pH meter. 0.5 g of gel was dissolved in 25 ml of distilled water and. Then the pH of each formulation was determined.

Spreadability

Spreadability is the area to which the gel spreads easily on application on any affected area of skin. 1gm of gel was placed on a glass slide and later second slide was placed varying the weights. The time taken for the separation of two slide and the distance by it was calculated.

Spreadability = <u>Weight tied to the upper side X length of the glass slides</u>
Time taken in seconds

Homogeneity

All developed Hydrogel were tested for homogeneity by visual inspection after the gels have been set in the container. They were tested for their appearance and presence of any aggregates**.**

Viscosity

Weigh 10–20 g of gel into the viscometer cup, remove air bubbles by gentle stirring/degassing, and equilibrate to 25 °C. Select an appropriate spindle (e.g., #2 or #3 for medium viscosity) and calibrate the Brookfield viscometer. Attach the spindle, set the required speed (10–100 rpm), and immerse it centrally in the sample. Start rotation and allow 30–60 seconds for stabilization. Record viscosity (cP) after at least 5 spindle rotations. Repeat measurements three times at different speeds to obtain rheological behavior. Average the readings, clean the spindle between runs, and compare results with specifications (e.g., USP <911>).

III. ANTIBACTERIAL SUSCEPTIBILITY ASSAY OF EEEUC AND EEEUCG1, EEUCG2, EEEUCG3 AGAINST MULTIDRUG-RESISTANT BACTERIAL STRAINS ISOLATED FROM DIABETIC FOOT ULCERS (DFU)

Effects of ethanolic extract of ***Eucalyptus camaldulensis*** leaves on diabetic foot ulcer evaluated by agar well diffusion method against **multidrug-resistant bacterial strains isolated from diabetic foot ulcers.** Collecting samples from diabetic foot ulcers at multiple levels requires sterile techniques to isolate multidrug resistant strain accurately. Deep samples like pus and bone are preferred over superficial swabs for reliable pathogen identification, as ***S. aureus*** is often predominant in diabetic foot infections**.**

Sample Collection and Microbiological Procedures

1. Sample Collection from Diabetic Foot Ulcers (DFU)

Superficial Swab:
After cleansing with sterile saline and debridement, the ulcer base was swabbed and transported in suitable medium for culture. This method reflects surface flora but may include contaminants.

Pus Sample:
Following sharp debridement, pus was aspirated aseptically or deep swab collected. Samples were transported in aerobic/anaerobic media within 2 hours. Deep cultures show good correlation with bone infections.

Bone Biopsy:
Percutaneous or intraoperative bone biopsy was performed for suspected osteomyelitis. Bone culture is the gold standard (95–99% accuracy) for pathogen identification (e.g., *Staphylococcus aureus*), followed by histopathology and susceptibility testing as per CLSI guidelines.

2. Primary Culture and Identification

Samples were streaked on Blood agar, Chocolate agar, and MacConkey agar and incubated at 35–37°C for 24–48 h.
Presumptive ***Staphylococcus aureus*** was identified by Golden yellow colonies (1–3 mm), Gram-positive cocci in clusters, Catalase and coagulase positivity. Subculturing was done using quadrant streaking to obtain pure isolates for further biochemical and antibiotic susceptibility testing.

Antibacterial Susceptibility Assay – Agar Well Diffusion

The agar well diffusion method was performed by first preparing sterile nutrient agar plates and uniformly inoculating them with the test microorganism using a sterile cotton swab. After solidification, wells were aseptically punched into the agar using a sterile cork borer. Measured volumes of the test sample, standard drug, and control were carefully introduced into the respective wells. The plates were then incubated at 37°C for 18–24 hours, after which the zones of inhibition were measured in millimeters to evaluate antimicrobial activity. Positive controls-Amoxiclav, Mupirocin and Negative control- Sterile distilled water. After 24 hr incubation at 37°C, zones of inhibition were measured[12]

IV. ANTIBIOFILM ASSAY OF EEEUCG1, EEEUCG2 and EEEUCG3 AGAINST MULTIDRUG-RESISTANT BACTERIAL STRAINS ISOLATED FROM DIABETIC FOOT ULCERS (DFU) – TUBE DILUTION METHOD

Biofilm biomass formed by ***S .aureus*** under static conditions was stained with crystal violet. Absorbance at 570 nm is proportional to biofilm formation. The test tube biofilm assay is a simple and widely used method to evaluate the ability of microorganisms to form biofilms on solid surfaces and to determine the antibiofilm activity of test compounds. In this method, bacterial cultures are grown in a nutrient medium under static conditions in sterile test tubes, which allows the cells to attach to the inner surface of the tube and produce a biofilm layer. Biofilms are structured microbial communities enclosed in an extracellular polymeric matrix composed of polysaccharides, proteins, and nucleic acids, which protect bacteria from environmental stress and antimicrobial agents. The principle of this method is based on surface attachment and staining of the biofilm biomass using crystal violet. After incubation, non-adherent cells are removed by washing, and the attached biofilm is fixed with methanol. Crystal violet binds to negatively charged components of bacterial cells and extracellular matrix present

in the biofilm. Excess stain is removed, and the bound dye is solubilized using acetic acid. The intensity of the resulting-colored solution is measured spectrophotometrically at 570 nm and is directly proportional to the amount of biofilm formed. A reduction in staining intensity or optical density in extract-treated samples compared to control indicates inhibition of biofilm formation and confirms antibiofilm activity of the test substance[13].

Procedure: To evaluate antibiofilm activity, a bacterial suspension (0.5 McFarland) was incubated with the extract at 37°C for 24 hours. Following incubation, the tubes were washed, fixed with methanol, and stained using 1% crystal violet. The bound stain was then solubilized with 30% acetic acid, and the optical density (OD) was measured at 570 nm. A reduction in OD compared to the control group served as the primary indicator of antibiofilm efficacy.

RESULT

The hydrogel formulation was prepared according to the standard procedure with slight modifications. The optimized concentration of ***Eucalyptus camaldulensis*** leaf extract was incorporated into a pre-hydrated Carbopol 934 gel base to obtain a uniform and stable topical formulation. The formulation was developed at various concentration of 1%, 2%, 3%, herbal based hydrogel and evaluated for their physical and quality parameters were prescribed in **table no:2**

The formulated Hydrogel was evaluated for the following parameters.

PARAMEERS	**Formulation-I (1%)**	**Formulation II (2%)**	**Formulation-III (3%)**
Clarity	Good	Good	Good
Uniformity	Good	Good	Good
Lumps	Absence	Absence	Absence
Phase seperation	Nil	Nil	Nil
Crack	Nil	Nil	Nil
Color	Pale green	Pale green	Olive green
Odor	Characteristic, aromatic odor	Characteristic, aromatic odor	Characteristic, aromatic odor
pH	6.1	6.3	6

Spreadability	4.5cm(good spreadability)	4.8cm(good spreadability)	4.9cm(good spreadability)
Homogeneity	Good	Good	Good
Viscosity(cP or dyne/cm)	1675	3300	4568

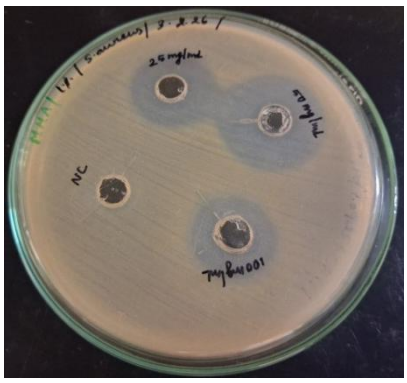
Table 2-Evaluation of EEEUCG parameters

The antibacterial susceptibility assay was performed in agar well diffusion method .The consequences of the study was observed and depicted in **table no.3 Fig.no.1**

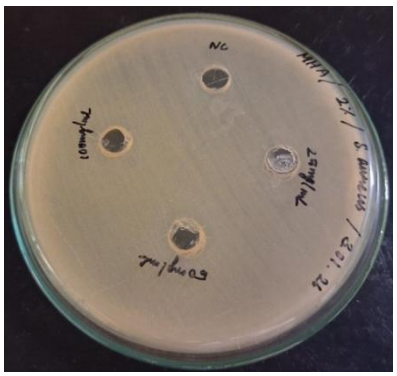
Placebo	**Ethanol**	**Standard drugs for DFU(mg/ M**		**Test Samples**			
No Zone	No Zone	**Std 1**	**Std 2**	**Conc mg/mL**	**EEEUCG1**	**EEEUCG2**	**EEEUCG3**
		38mm	42mm	**25**	21mm	No Zone	No Zone
				50	26mm	No Zone	No Zone
				100	20mm	No Zone	No Zone

Table no-3: Antibacterial Susceptibility Assay Of EEEUCG (1-3)

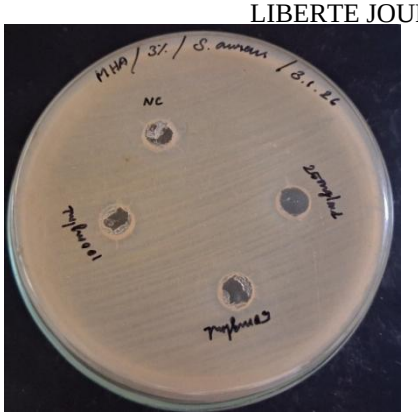
Std 1- Amoxiclav **Std 2**- Mupirocin, **EEEUCG-**EEEUC **Gel (1%, 2%, 3%)**

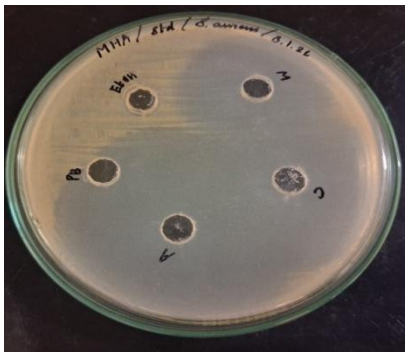


a)EEEUCG1



b)EEEUCG2





c)EEEUCG3 d)Crude Extract

Fig.no.1- **Antibacterial Susceptibility Assay Of EEEUCG(1-3)**

The antimicrobial efficacy of the test samples was assessed by determining the **zone of inhibition (mm)** against the selected diabetic foot ulcer (DFU) pathogen using the agar diffusion assay. The **placebo and ethanol control exhibited no detectable inhibitory zone**, confirming the absence of intrinsic antimicrobial activity of the solvent and validating the reliability of the experimental system.

Among the evaluated fractions, **EEEUCG1 exhibited discernible antibacterial activity** across the tested concentrations. At **25 mg/mL**, a measurable inhibition zone of **21 mm** was observed, which increased to **26 mm at 50 mg/mL**, indicating enhanced antibacterial potency at this concentration. However, at **100 mg/mL**, the inhibition zone marginally declined to **20 mm**.

Conversely, **EEEUCG2 and EEEUCG3 failed to exhibit any inhibitory effect**, as no zones of inhibition were recorded at any of the tested concentrations.

The antibiofilm activity was performed on crude extract and Gel formulation. The consequences of the study was observed and depicted in **Table no:4 and fig.no:2**

Biofilm Inhibition (%) = (OD Control-OD Test)÷(OD Control) ×100

EXRACRT: OD_{570nm} for control (Growth control) = 0.54

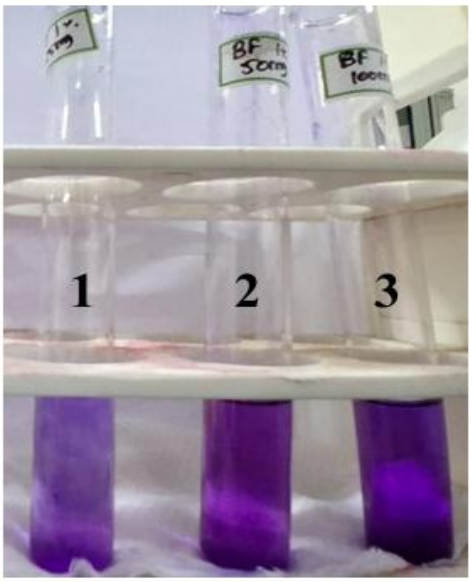
GEL: OD_{570nm} for control (Growth control) = 0.48

Blank = Sterile Nutrient Broth (Sterility control)

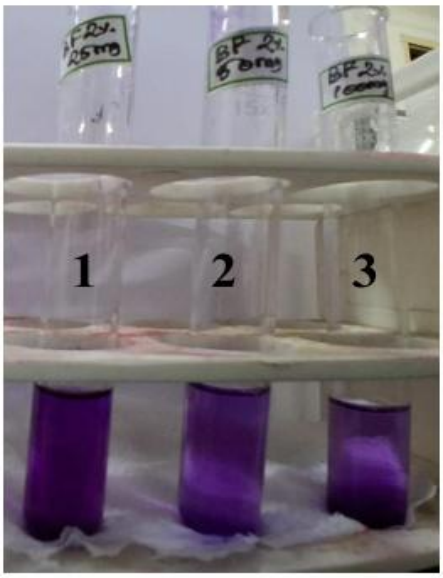
S.No	Test sample (EEEUCG)	Concentration(mg/ml)	OD at 570 nm	% Biofilm inhibition
1.	1%	25	0.18	62.5
		50	0.12	75
		100	0.03	93.75
2.	2%	25	0.19	60.41
		50	0.13	72.91
		100	0.12	75
3.	3%	25	0.18	62.5
		50	0.13	72.91
		100	0.10	79.16
4.	Std 1	5	0.01	99.9
5.	Std 2	5	0.01	99.9

Table no.-4: Anti Biofilm Activity Of EEEUCG (1-3) Against Multidrug-Resistant Bacterial Strains Isolated From Diabetic Foot Ulcers (DFU)

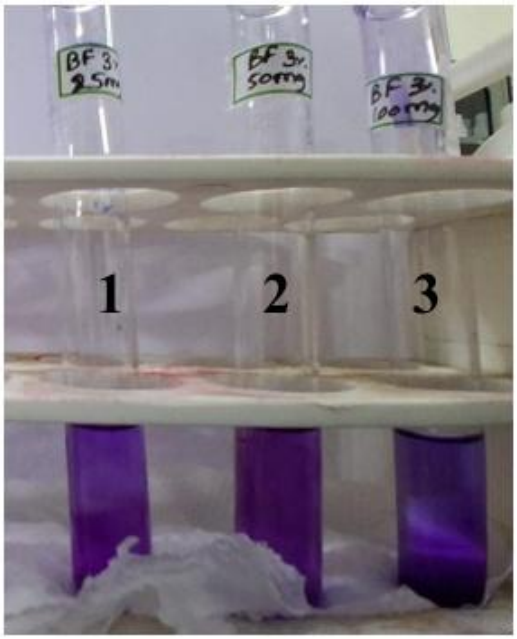
Std 1-Linezolid, **Std 2-**Silversulphadiazine ointment, **EEEUCG (1%, 2%, 3%)**



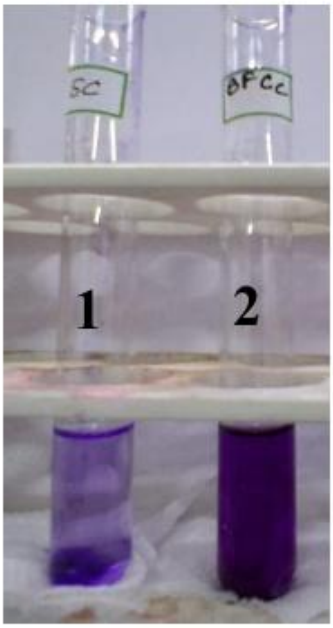
1% Gel Formulation
1- 25mg/mL, 2. 50mg/mL, 3. 100mg/mL



2% Gel Formulation
1- 25mg/mL, 2. 50mg/mL, 3. 100mg/mL



3% Gel Formulation
1- 25mg/mL, 2. 50mg/mL, 3. 100mg/mL



1- Sterility Control (Blank)
2- Culture Control

Fig.No:4- Anti Biofilm Activity Of **EEEUCG (1-3) Against Multidrug-Resistant Bacterial Strains Isolated From Diabetic Foot Ulcers (DFU)**

The figure represents the qualitative analysis of biofilm formation. The intensity of violet coloration is directly proportional to the extent of biofilm produced by the test organism ***Staphylococcus aureus.***

The antibiofilm activity of the crude extract, Linezolid, and Silversulphadiazine ointment was evaluated by the test tube crystal violet assay. The optical density (OD) of the growth control was 0.54, while sterile nutrient broth served as the blank.

The crude extract showed a significant reduction in biofilm formation at all tested concentrations. At 25 mg/mL, the crude extract showed 94.4% inhibition of biofilm formation. At higher concentrations (50 mg/mL and 100 mg/mL), the extract exhibited maximum inhibition (99.9%), indicating strong antibiofilm activity in a concentration-dependent manner.

Similarly, Linezolid and Silversulphadiazine ointment at 5 mg/mL showed very low optical density values (0.01) with 99.9% biofilm inhibition, demonstrating excellent antibiofilm activity.

The reduction in optical density in extract-treated samples compared to the growth control indicates inhibition of biofilm formation. The crude extracts showed strong antibiofilm activity, which increased with increasing concentration. The highest inhibition was observed at 50 mg/mL and 100 mg/mL, suggesting that the extract effectively prevents bacterial adhesion and biofilm development.

Linezolid and Silversulphadiazine ointment exhibited maximum inhibition (99.9%), indicating their high effectiveness as antibiofilm agents. The results suggest that the test samples interfere with biofilm formation by reducing microbial attachment and extracellular matrix production. Based on the percentage inhibition values (>75%), all tested samples showed strong antibiofilm activity.

DISCUSSION:

The present investigation reveals that the EEEUCG1 fraction of the ethanolic extract possesses appreciable antibacterial potential against the DFU-associated pathogen, whereas EEEUCG2 and EEEUCG3 demonstrated no observable antimicrobial efficacy under the tested experimental conditions. The absence of inhibition in the placebo and ethanol controls unequivocally confirms that the antimicrobial activity observed is attributable to the bioactive phytoconstituents present within the extract fraction.

Notably, the maximum inhibitory activity was recorded at 50 mg/mL (26 mm), suggesting that this concentration may represent an optimal diffusion and bioactivity threshold for the extract within the agar matrix. The slight reduction in inhibition at 100 mg/mL could plausibly be attributed to reduced diffusibility of highly concentrated phytochemical constituents within the agar medium, which may limit their effective antimicrobial interaction.

Although the inhibitory zones produced by EEEUCG1 were comparatively lower than those exhibited by the standard antibiotics (38–42 mm), the extract nevertheless demonstrated considerable antibacterial activity, indicating the presence of pharmacologically active phytochemicals. This activity may be associated with phenolic compounds, flavonoids, and terpenoid constituents, which are widely reported to exert antimicrobial effects through mechanisms such as disruption of bacterial cell membranes, inhibition of enzymatic systems, and interference with microbial metabolic pathways [5,14].

Collectively, these findings suggest that the ethanolic extract fraction of ***Eucalyptus camaldulensis*** (EEEUCG1) represents a promising source of natural antimicrobial agents with potential therapeutic relevance in the management of diabetic foot ulcer infections. However, further investigations, including minimum inhibitory concentration (MIC) determination, biofilm inhibition assays, and mechanistic studies, are warranted to comprehensively elucidate its antimicrobial potential and clinical applicability.

Staphylococcus aureus–mediated biofilm formation represents the quintessential pathogenic paradigm underpinning the persistence and recalcitrance of diabetic foot infections (DFIs). Within the poly microbial milieu of diabetic foot ulcers (DFUs), ***S. aureus*** frequently orchestrates a highly organized, sessile microbial consortium encased in a self-produced extracellular polymeric substance (EPS), thereby establishing a formidable defensive niche[10,11,12]. This biofilm phenotype engenders profound antimicrobial tolerance and immune evasion, effectively insulating bacterial aggregates from phagocytic clearance, oxidative stress, and conventional antibiotic regimens. Empirical observations have demonstrated that DFU-derived biofilm isolates necessitate antibiotic concentrations 10–1000-fold greater than their planktonic counterparts to achieve microbial eradication, underscoring their extraordinary therapeutic resilience [11].

The epidemiological burden of biofilm-associated pathology in chronic DFUs is substantial, with prevalence estimates ranging from 60% to 80% [11]. Biofilm production has been widely reported among DFU isolates and is strongly associated with increased antimicrobial resistance and delayed wound healing [2,10]. Furthermore, microscopy-based studies have substantiated the presence of structurally mature biofilms even in clinically superficial wounds, thereby challenging conventional clinical assessments of infection severity [12].

Supplementary investigations have demonstrated statistically significant associations between robust biofilm formation, inadequate glycemic control, and delayed epithelialization. Collectively, these data delineate biofilm formation not merely as an ancillary virulence determinant but as a central pathogenic axis driving chronic inflammation, delayed tissue regeneration, and escalating antimicrobial resistance. Consequently, the strategic prioritization of anti-biofilm therapeutics targeting ***S. aureus*** is both mechanistically substantiated and clinically imperative [6,10].

The crude extract demonstrated pronounced antibiofilm activity at all tested concentrations. At 25 mg/mL, it produced 94.4% inhibition, while higher concentrations (50 and 100 mg/mL) achieved near-complete inhibition (99.9%), indicating strong concentration-dependent activity and suggesting effective interference with microbial adhesion and extracellular matrix development. Similarly, Linezolid and Silver sulfadiazine ointment exhibited excellent antibiofilm activity, confirming their effectiveness as reference standards.

The gel formulations also showed significant inhibitory effects against ***Staphylococcus aureus.*** The 1% gel exhibited the highest efficacy, showing near-complete suppression of biofilm formation and superior performance among the formulations. The 2% gel demonstrated a plateau effect at higher concentrations, while the 3% gel showed moderate to strong inhibition but remained less effective than the 1% gel.

Overall, all tested samples demonstrated strong antibiofilm activity, with percentage inhibition values exceeding 75% at higher concentrations. Biofilm inhibition increased with concentration across treatments, indicating dose-dependent effects. These findings are consistent with previous literature, although slightly higher inhibition values were observed compared to earlier reports[13].

From the above findings, it was observed that the extract and gel formulation significantly reduced biofilm biomass and disrupted EPS matrix integrity. Active phytoconstituents such as 1,8-cineole and phenolic compounds likely inhibit quorum sensing mechanisms, thereby preventing structured biofilm formation[5]. The antibiofilm activity also addresses persistent infections caused by small colony variants (SCVs), a major factor in DFU recurrence and antibiotic failure[11].

CONCLUSION

The present study successfully developed a topical gel formulation containing the ethanolic extract of ***Eucalyptus camaldulensis*** and evaluated its antibacterial and antibiofilm potential against DFU-associated pathogens. The formulated gels exhibited satisfactory physicochemical properties suitable for topical application. The antibacterial assay confirmed inhibitory activity against the clinical isolate of ***Staphylococcus aureus*** obtained from diabetic foot ulcer samples, while the antibiofilm study demonstrated significant inhibition of biofilm formation, particularly with the 1% gel formulation. Although the activity was comparatively lower than the standard drugs Linezolid and Silver sulfadiazine, the results indicate promising therapeutic potential. Further investigations including in vivo wound healing studies and formulation optimization are recommended to validate the clinical applicability of the developed gel formulations. The 1% concentration demonstrated superior performance compared to other formulations.

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