

Formulation, Antimicrobial Evaluation and Stability Kinetics of a Polyherbal Leather Insole Containing *Bryophyllum pinnatum*, *Calendula officinalis* and *Althaea officinalis* Extracts

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Abstract: Infected diabetic foot ulcers (DFUs) are a leading cause of non-traumatic lower-limb amputation, and rising antimicrobial resistance is limiting conventional antibiotic therapy. A leather insole that carries plant-derived antimicrobials and sits in continuous contact with the plantar surface could complement standard wound care. We extracted leaves of *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis* sequentially with *n*-hexane, dichloromethane, ethyl acetate, and ethanol, and screened the 12 extracts for phytochemicals. We profiled the ethanol extracts by GC-MS. The antimicrobial activity of the extracts, and of vegetable-tanned leather (VTL) loaded with them, was tested by agar disc diffusion, MIC and MBC/MFC against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Candida albicans* isolated from DFU patients. A polyherbal insole (1:1:1, 25 mg/mL) was characterised by FTIR, and its antimicrobial potency was monitored for 96 days and fitted to first-order decay kinetics. Steroids, cardiac glycosides and terpenoids were present in all 12 extracts, and the more polar extracts had the richest profiles. Fatty acid methyl esters dominated the GC-MS profiles; oleic acid methyl ester made up 42.3% of the *C. officinalis* extract and 20.8% of the *B. pinnatum* extract. The extracts produced inhibition zones of 18–42 mm and MICs of 0.10–1.25 mg/mL, with an MBC/MIC ratio of 2 in every case, which indicates cidal activity. *C. officinalis* was the most active extract against *S. aureus* (42 mm; MIC 0.10 mg/mL), outperforming ciprofloxacin (28 mm), and all three extracts exceeded fluconazole against *C. albicans* (33–37 mm vs 20 mm). Extract-

loaded leather retained on average 77% of the free-extract zone of inhibition. FTIR band shifts (O-H 3360 $\rightarrow$ 3297 cm^{-1} ; C=O 1709 $\rightarrow$ 1638 cm^{-1}) indicated hydrogen bonding between the extract and the collagen matrix. The inhibition zone of the polyherbal insole fell from 42 mm to 16 mm by Day 84 and then plateaued. Regression across Days 0–84 gave $k = 0.0116 \text{ day}^{-1}$ ($R^2 = 0.981$), $t_{1/2} \approx 60$ days and $t_{90} \approx 9$ days. The three extracts have broad antibacterial and anticandidal activity that largely survives incorporation into leather. The polyherbal insole retains useful activity for about two months, but its short t_{90} suggests replacing the insole at defined intervals or improving storage conditions.

Keywords: diabetic foot ulcer; *Bryophyllum pinnatum*; *Calendula officinalis*; *Althaea officinalis*; antimicrobial insole; vegetable-tanned leather; shelf life; first-order kinetics

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1.0 Introduction

Diabetic foot ulcers (DFUs) are among the most serious complications of diabetes mellitus, a disease that affected an estimated 537 million adults worldwide in 2021 (Sun *et al.*, 2022). They arise from the combined effects of peripheral neuropathy, peripheral arterial disease, impaired tissue repair, and repeated mechanical stress on an insensate foot (Armstrong *et al.*, 2023). An estimated 19–34% of people with diabetes will develop a foot ulcer during their lifetime, about half of these ulcers become infected, and a substantial proportion of moderate or severe infections may result in lower-extremity amputation (Armstrong *et al.*, 2017, 2023). The global prevalence of DFU has been estimated at approximately 6.3%, with a higher prevalence reported in Africa than in several other regions (Zhang *et al.*, 2017). Impaired immunity in diabetes, including dysregulated neutrophil and macrophage function and persistent inflammation, further delays wound healing and increases susceptibility to infection (Dawi *et al.*, 2025). Consequently, DFU management requires a multidisciplinary approach that addresses infection, wound care, pressure off-loading, vascular status and metabolic control rather than antimicrobial treatment alone (Senneville *et al.*, 2024).

Staphylococcus aureus is among the organisms most frequently associated with diabetes-related foot infections. Gram-negative organisms such as *Pseudomonas aeruginosa* and *Escherichia coli* may also occur, particularly in chronic or previously treated wounds (Senneville *et al.*, 2024). Hospital-based studies in Nigeria have reported these organisms among isolates from infected DFUs, with multidrug resistance representing an important therapeutic concern (Ako-Nai *et al.*, 2006; Adeyemo *et al.*, 2021). Fungi, including *Candida* species, may also occur in chronic

wound environments, and fungal communities have been associated with delayed healing (Kalan *et al.*, 2016). In Nigeria and other low- and middle-income settings, inappropriate and poorly regulated antibiotic use has contributed to antimicrobial-resistance concerns (Olagunju *et al.*, 2025). Globally, bacterial antimicrobial resistance was associated with an estimated 4.95 million deaths in 2019, with a particularly high burden reported in western sub-Saharan Africa (Murray *et al.*, 2022). The continuing burden of resistant microorganisms therefore creates a need to investigate complementary antimicrobial materials that could provide localized antimicrobial functionality while being used alongside established DFU prevention and clinical management strategies. Such materials should not, however, be regarded as substitutes for clinically indicated systemic antimicrobial therapy or other evidence-based DFU care.

Medicinal plants are a promising source of bioactive compounds with potential antimicrobial and wound-related applications. Their extracts contain chemically diverse constituents that may act through multiple biological mechanisms, and several medicinal plants have a long history of traditional use in wound care (Sofowora, 1993; Cowan, 1999; Miraj *et al.*, 2023). The use of chemically diverse plant extracts is particularly relevant to the present study because a polyherbal formulation may provide a broader phytochemical profile than a preparation based on a single plant, although the biological performance and stability of such combinations must be experimentally established rather than assumed.

Bryophyllum pinnatum (Lam.) Oken (Crassulaceae) contains bufadienolides, flavonoids such as quercetin and kaempferol, and triterpenoid constituents and has been investigated for wound-healing, anti-inflammatory and antimicrobial activities (Akinsulire *et al.*, 2007; Nayak *et al.*, 2010; Al Snafi, 2013; Fürer *et al.*, 2016; Andrade *et*



al., 2020; Isibor, 2026). *Calendula officinalis* L. (Asteraceae) contains several classes of bioactive compounds and has been investigated for antimicrobial and wound-related applications. Its triterpenoid constituents and essential oils have demonstrated biological activities, while clinical and experimental studies have examined its potential in wound management (Zitterl-Eglseer *et al.*, 1997; Gazim *et al.*, 2008; Efstratiou *et al.*, 2012; Parente *et al.*, 2012; Buzzi & de Freitas, 2016; Givol *et al.*, 2019; Shahane *et al.*, 2023). Evidence from a systematic review indicates that *C. officinalis* has been investigated in both experimental and clinical wound-healing studies, although the available evidence is heterogeneous and does not establish it as a definitive treatment for diabetic ulcers (Givol *et al.*, 2019). *Althaea officinalis* L. (Malvaceae), commonly known as marshmallow, contains mucilage polysaccharides, phenolic compounds and flavonoids. These constituents have been investigated for protective, anti-inflammatory and antioxidant effects (Bonaterra *et al.*, 2020; Bahari *et al.*, 2025). Its leaf extract has demonstrated antibacterial and wound-healing activity in an experimental excision-wound model (Rezaei *et al.*, 2015), while its polysaccharides have been reported to influence the physiology of human epithelial cells *in vitro* (Deters *et al.*, 2010).

Although these three plants have independently attracted attention for their phytochemical and biological properties, their combination in a single leather-based delivery system presents a different research problem. The antimicrobial activity observed in a free plant extract cannot automatically be assumed to persist after incorporation into a solid material. Extraction solvent, phytochemical composition, concentration, interactions among plant constituents, interactions with the leather matrix, and the accessibility of active compounds at the material surface may all influence the final antimicrobial performance. Therefore, formulation and post-incorporation

evaluation are essential steps in determining whether plant extracts can function effectively as components of an antimicrobial leather material.

How an active agent is delivered to the target site is an important consideration in developing antimicrobial materials. Wound dressings and other delivery platforms, including electrospun systems capable of releasing natural extracts, have been investigated for localized delivery applications (Jiang *et al.*, 2023; Fiaschini *et al.*, 2024). Insoles are already important components of diabetic-foot care because appropriately designed footwear and off-loading devices can help redistribute plantar pressure and reduce mechanical loading (Collings *et al.*, 2023; Tang *et al.*, 2023; Bus *et al.*, 2024). This makes the insole an attractive material platform for investigating additional functions that could complement, rather than replace, mechanical protection. Because an insole remains in close proximity to the plantar surface for prolonged periods during use, it provides a potentially suitable substrate for localized antimicrobial functionality. Previous studies have demonstrated antimicrobial properties in modified leather and other functional insole systems. Silver-based coatings on leather insoles have shown antibacterial and antifungal activity (Marques *et al.*, 2022; Carvalho *et al.*, 2022), while BaTiO₃-based composite insoles have been investigated for antibacterial activity and infected-wound applications (Liu *et al.*, 2024).

Despite these developments, three important issues remain insufficiently addressed. First, most studies on the selected medicinal plants have evaluated the extracts themselves rather than their incorporation into vegetable-tanned leather as a footwear material. Second, antimicrobial activity demonstrated by a free extract does not necessarily represent the activity retained after immobilization or interaction with a leather matrix. Third, even when an antimicrobial material initially demonstrates strong activity, its practical usefulness depends on



how rapidly that activity changes during storage. Consequently, information on the formulation of plant-extract-loaded leather, the retention of antimicrobial activity following incorporation, and the kinetics of antimicrobial potency loss remains limited.

The stability question is particularly important for a formulated antimicrobial insole. A material intended for practical use should retain an adequate level of antimicrobial activity for a defined period under specified storage conditions. Loss of activity may result from degradation, oxidation, volatilization, chemical interaction with the leather matrix, changes in extract availability, or other physicochemical processes. Monitoring antimicrobial activity at defined storage intervals and fitting the resulting data to an appropriate kinetic model can therefore provide quantitative information about the rate of potency loss. Parameters such as the apparent first-order rate constant, half-life, and time required for a specified percentage loss of activity can provide a more meaningful basis for discussing stability than a single antimicrobial measurement at the beginning or end of storage.

The application of kinetic modelling to an antimicrobial leather insole also provides a direct connection between formulation science and product-use considerations. If antimicrobial activity declines according to an identifiable kinetic pattern, the resulting model can help characterize the useful activity period of the material under the conditions investigated. Such estimates are formulation- and storage-condition dependent and should not be interpreted as clinical expiry periods without further validation. Nevertheless, they provide an important preliminary basis for optimizing extract concentration, polyherbal composition, packaging, storage conditions, and replacement intervals.

Accordingly, the principal knowledge gap addressed by the present study lies at the intersection of phytochemistry, antimicrobial science, leather technology and stability

assessment. Existing literature provides evidence for the biological activities of *B. pinnatum*, *C. officinalis* and *A. officinalis*, while other studies have demonstrated that leather and other footwear materials can be modified to possess antimicrobial properties. However, limited information is available on a polyherbal formulation incorporating these three medicinal plants into vegetable-tanned leather, the antimicrobial activity retained after incorporation, the physicochemical interaction between the extracts and leather matrix, and the quantitative kinetics governing the decline in antimicrobial activity during storage. Addressing these issues is necessary for determining whether such a formulation has sufficient material and antimicrobial characteristics to justify further development.

The present study therefore aimed to formulate, characterize and evaluate a polyherbal antimicrobial leather insole containing *B. pinnatum*, *C. officinalis* and *A. officinalis* extracts. Specifically, the study sought to: (i) characterize the phytochemical composition of extracts obtained using solvents of different polarities; (ii) identify major chemical constituents of the selected ethanol extracts using gas chromatography–mass spectrometry (GC-MS); (iii) determine the antimicrobial activity of the extracts against selected bacterial and fungal isolates obtained from DFU patients; (iv) incorporate the extracts individually and in combination into vegetable-tanned leather; (v) evaluate the antimicrobial activity retained by the extract-loaded leather; (vi) characterize extract–leather interactions using Fourier-transform infrared (FTIR) spectroscopy; and (vii) monitor the antimicrobial activity of the optimized polyherbal leather insole during storage and apply kinetic modelling to estimate its rate of activity loss, apparent half-life and stability period under the experimental conditions.

The significance of this study is both scientific and technological. Scientifically, it provides comparative information on the phytochemical composition and



antimicrobial performance of three medicinal plants and examines whether their biological activity is retained following incorporation into a leather matrix. The FTIR investigation further provides evidence concerning possible chemical or intermolecular interactions between extract constituents and the collagen-based leather substrate. The stability study extends the antimicrobial evaluation beyond initial activity by providing quantitative information on the temporal loss of potency.

From a materials-development perspective, the study explores the possibility of converting vegetable-tanned leather from a conventional footwear substrate into a multifunctional material with an additional antimicrobial property. Such functionality could potentially complement established pressure-off-loading and footwear strategies for people at risk of DFU, while the stability data may assist subsequent optimization of formulation and storage conditions. The study does not establish clinical efficacy in DFU treatment, nor does it imply that an antimicrobial insole can replace wound care, off-loading, antimicrobial therapy or other clinically indicated interventions. Rather, it provides a materials and formulation basis for subsequent investigations involving release characteristics, cytocompatibility, skin compatibility, mechanical performance, pressure redistribution, durability, moisture management, in vivo evaluation and, ultimately, controlled clinical studies.

Overall, the study addresses the need for a systematic assessment of not only whether the selected plant extracts possess antimicrobial activity, but also whether that activity can be incorporated into a leather insole, retained after formulation, characterized chemically, and maintained for a measurable period during storage. This integrated approach provides a stronger basis for evaluating the feasibility of polyherbal leather as a multifunctional antimicrobial footwear material.

2.0 Materials and Methods



2.1 Collection and Processing of Plant Materials

Fresh leaves of *Bryophyllum pinnatum* (Lam.) Oken, *Calendula officinalis* L., and *Althaea officinalis* L. were collected from Zaria, Kaduna State, Nigeria. The plant materials were authenticated at the Herbarium Unit, Department of Botany, Ahmadu Bello University, Zaria, Nigeria. Voucher specimens were deposited under the numbers ABUH01894, ABUH09024, and ABUH01641 for *B. pinnatum*, *C. officinalis*, and *A. officinalis*, respectively.

The collected leaves were inspected to remove extraneous materials and visibly damaged portions. They were washed three times with tap water and subsequently rinsed twice with distilled water to remove adhering soil and other contaminants. The leaves were air-dried at room temperature under shade to minimize possible degradation of heat- or light-sensitive phytoconstituents. The dried materials were pulverized using a clean mortar and pestle, passed through a No. 40 sieve to obtain relatively uniform particle sizes, and stored in airtight containers until extraction.

Fig. 1 shows representative specimens of the three medicinal plants used in the study.

2.2 Preparation of Plant Extracts

The powdered leaves of *B. pinnatum*, *C. officinalis*, and *A. officinalis* were separately extracted by maceration using ethanol, ethyl acetate, dichloromethane, and *n*-hexane as extraction solvents. Using solvents spanning a broad polarity range aimed to recover chemically diverse phytoconstituents and to enable comparison of the phytochemical profiles and antimicrobial properties of extracts obtained under different solvent conditions.

Following maceration, the respective mixtures were filtered, and the filtrates were concentrated under reduced pressure using a rotary evaporator (Büchi, model V-700, Switzerland) at a temperature below 50 °C. The concentrated extracts were then air-dried to obtain the respective crude extracts. The dried extracts were weighed, transferred into



appropriately labelled airtight containers, and stored under suitable conditions until further analysis.

The extraction procedure produced twelve crude extracts, comprising four solvent fractions from each of the three plant species. The extracts were subsequently subjected to preliminary phytochemical screening and antimicrobial evaluation. Based on their phytochemical richness and antimicrobial performance, the ethanol extracts were selected for further chemical profiling by gas chromatography–mass spectrometry (GC–MS).

2.3 Preliminary Phytochemical Screening

The twelve crude extracts obtained from the three plant species were screened qualitatively for major classes of secondary metabolites using standard phytochemical screening procedures. The screening was performed to establish the broad chemical composition of the extracts and to identify metabolite classes potentially associated with the observed antimicrobial activity.

The phytochemical screening covered alkaloids, cardiac glycosides, saponins, phenolics, tannins, steroids, carbohydrates, flavonoids, terpenoids, and anthraquinones. Appropriate qualitative tests were performed for each metabolite class, and results were recorded based on the presence or absence of the characteristic reaction.

The phytochemical profiles were subsequently compared among the different extraction solvents and plant species to determine whether solvent polarity influenced the diversity of metabolites recovered.

2.4 Gas Chromatography–Mass Spectrometry Analysis

The ethanol extracts of *B. pinnatum*, *C. officinalis*, and *A. officinalis* were subjected to gas chromatography–mass spectrometry (GC–MS) analysis to characterize their volatile and semi-volatile chemical constituents. This analysis complemented the preliminary phytochemical screening by providing compound-level information on

the major constituents detected in the selected extracts.

We identified the chromatographic peaks by comparing their mass spectra with appropriate reference spectral libraries and/or available compound databases. The relative abundance of identified compounds was expressed as a percentage of the total chromatographic composition. Particular attention was given to fatty acid derivatives and other constituents occurring at relatively high proportions because of their potential contribution to the biological properties of the extracts.

The GC–MS profiles were used to identify the major constituents reported for the three extracts, including oleic acid methyl ester, methyl palmitate, and eicosane.

2.5 Preparation and Characterization of Test Microorganisms

The antimicrobial evaluation was performed against selected microorganisms associated with diabetic foot-ulcer infection. The test organisms comprised *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Candida albicans*. The organisms were obtained from diabetic foot-ulcer clinical isolates as specified for the study.

The isolates were maintained under appropriate microbiological conditions and subcultured before antimicrobial testing to obtain viable cultures. Standardized inocula were prepared for the antimicrobial assays to ensure comparability among the different plant extracts and leather formulations.

2.6 Evaluation of Antimicrobial Activity of Plant Extracts

The antimicrobial activity of the crude plant extracts was initially evaluated using the agar disc-diffusion method. Appropriate quantities of each extract were placed on sterile discs, which were then placed on agar media previously inoculated with the respective test microorganisms. Suitable controls were included to interpret the antimicrobial effects attributable to the plant extracts.



Following incubation under appropriate conditions for the respective microorganisms, the diameter of each zone of inhibition was measured in millimetres. The inhibition-zone measurements were used to compare the antimicrobial potency of the extracts against the bacterial and fungal test organisms.

The resulting inhibition zones ranged from 18–42 mm across the extracts and test organisms. These measurements were subsequently used to identify the most active extracts for incorporation into the leather substrate.

2.7 Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of the plant extracts was determined to establish the lowest concentration capable of visibly inhibiting the growth of each test microorganism. Serial concentrations of the respective extracts were prepared and tested against standardized microbial inocula using an appropriate broth dilution procedure.

After incubation, the tubes or wells were examined for visible microbial growth. The MIC was recorded as the lowest extract concentration at which visible growth was inhibited. The MIC values were expressed in mg/mL.

The observed MIC values ranged from 0.10 to 1.25 mg/mL, with *C. officinalis* producing a particularly low MIC of 0.10 mg/mL against *S. aureus*.

2.8 Determination of Minimum Bactericidal and Fungicidal Concentrations

The minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) was determined from concentrations showing no visible growth in the MIC assay. Aliquots from the appropriate concentrations were subcultured onto fresh, extract-free growth media and incubated under suitable conditions. The lowest concentration that prevented subsequent microbial growth was recorded as the MBC for bacterial organisms or MFC for *C. albicans*.

The relationship between MBC/MFC and MIC was used to characterize the nature of the antimicrobial effect. An MBC/MIC ratio

of approximately 2, as observed in the study, was interpreted as evidence of a cidal rather than merely inhibitory effect under the experimental conditions.

2.9 Preparation of Extract-Enriched Vegetable-Tanned Leather

Vegetable-tanned leather was used as the substrate for incorporation of the selected plant extracts. Leather specimens were prepared into suitable test pieces and cleaned to remove surface contaminants before extract incorporation. The leather was subsequently treated with the individual plant extracts and the combined polyherbal formulation.

The extracts were incorporated into the leather substrate at the concentration used for the antimicrobial formulation. Individual extract-loaded leather specimens were prepared for *B. pinnatum*, *C. officinalis*, and *A. officinalis*, while a combined formulation containing the three extracts was prepared at a 1:1:1 ratio.

The incorporation procedure was designed to permit interaction between the plant-derived constituents and the collagen-based leather matrix while retaining sufficient extract at or near the leather surface for subsequent antimicrobial evaluation. Following treatment, the leather specimens were conditioned under the specified experimental conditions before characterization and antimicrobial testing.

2.10 Formulation of the Polyherbal Leather Insole

The polyherbal formulation was prepared by combining the selected extracts of *B. pinnatum*, *C. officinalis*, and *A. officinalis* in a 1:1:1 ratio. The combined extract was formulated at 25 mg/mL and incorporated into vegetable-tanned leather to produce the polyherbal antimicrobial insole.

The 1:1:1 composition was selected to provide an equal contribution from each of the three medicinal plants and to facilitate evaluation of the combined antimicrobial performance. The formulated leather was



subsequently subjected to antimicrobial testing and FTIR characterization.

2.11 Antimicrobial Evaluation of Extract-Enriched Leather

The antimicrobial activity of the extract-enriched leather specimens was evaluated using the same agar diffusion principle applied to the crude extracts. Leather discs or appropriately sized leather specimens containing the plant extracts were placed in contact with agar surfaces inoculated with the test microorganisms.

After incubation, the diameter of the resulting inhibition zones was measured in millimetres. The antimicrobial activity of extract-loaded leather was compared with that of the corresponding free extracts to determine the extent to which antimicrobial potency was retained following incorporation into the leather matrix.

The retention of activity was expressed by comparing the inhibition-zone diameter obtained from the extract-loaded leather with that obtained from the corresponding free extract under comparable test conditions.

2.12 Fourier-Transform Infrared Spectroscopy

Fourier-transform infrared (FTIR) spectroscopy was used to characterize the chemical functional groups present in the untreated leather and extract-enriched leather and to investigate possible interactions between the plant extracts and the leather matrix. Untreated leather and extract-containing leather specimens were subjected to FTIR analysis over the relevant infrared spectral region.

The characteristic absorption bands were assigned to functional groups based on their observed wavenumbers and comparison with established infrared assignments. Particular attention was given to the O–H, C–H, C=O, C–O and N–H regions because changes in these bands could indicate interactions between extract constituents and functional groups within the collagen-containing leather matrix.

Differences in band position and intensity between untreated and extract-loaded leather were evaluated to provide evidence for the incorporation of plant-derived constituents and possible intermolecular interactions.

2.13 Stability Study of the Polyherbal Leather Insole

The storage stability of the optimized polyherbal leather insole was assessed by monitoring its antimicrobial activity at predetermined storage intervals. The inhibition-zone diameter was used as the analytical response because it provides a direct measure of the antimicrobial activity retained by the formulated leather during storage.

The antimicrobial activity was monitored over the specified study period, including measurements extending to 96 days. At each sampling point, representative portions of the polyherbal leather formulation were subjected to the antimicrobial assay under comparable conditions. The inhibition-zone diameter obtained at each time point was recorded and compared with the initial activity.

The stability assessment was designed to determine whether the antimicrobial activity of the formulated leather declined progressively during storage and to establish the approximate period over which useful antimicrobial activity was retained.

2.14 Stability-Kinetic Analysis

The decrease in antimicrobial activity during storage was analysed using a first-order kinetic model. The change in antimicrobial activity with time was represented using the general first-order relationship expressed as equation 1

$$\ln \left(\frac{A_t}{A_0} \right) = -kt \quad (1)$$

where (A_0) represents the initial antimicrobial activity, (A_t) is the antimicrobial activity remaining at time (t) , (k) is the apparent first-order degradation or activity-loss rate constant, and (t) is the storage time.

The apparent rate constant was obtained from the slope of the linear relationship between $(\ln(A_t/A_0))$ and storage time. The



coefficient of determination (R^2) was used to assess the adequacy of the first-order model in describing the observed decline in antimicrobial activity.

The apparent half-life ($t_{1/2}$) was calculated using equation 2

$$t_{1/2} = 0. \frac{693}{k} \quad (2)$$

The time required for 90% retention or 90% loss, as defined by the stability criterion applied to the experimental data, was calculated from the corresponding first-order relationship. These kinetic parameters provided a quantitative description of the temporal stability of the antimicrobial leather formulation, rather than relying solely on qualitative observations of activity loss.

2.15 Data Analysis

The antimicrobial results were expressed as inhibition-zone diameters in millimetres, while MIC and MBC/MFC values were reported in mg/mL. We compared the antimicrobial performance of the free extracts with that of the corresponding extract-enriched leather formulations. We expressed GC–MS results as the relative percentage composition of the identified compounds, and interpreted FTIR results from characteristic absorption bands and their changes after extract incorporation.

For the stability experiment, antimicrobial activity was plotted against storage time and fitted to the selected first-order kinetic model. The calculated rate constant, coefficient of determination, half-life and stability-related time parameter were used to characterize the loss of antimicrobial potency during storage.

3. 0 Results and Discussion

3.1 Results

Plant collection and authentication (voucher nos. ABUH01894, ABUH09024, ABUH01641), sequential extraction, phytochemical tests, isolation and identification of organisms, disc diffusion/MIC/MBC, insole preparation, FTIR, GC-MS, stability study and kinetic equations.] 3.1 Extraction yield and phytochemical screening Sequential extraction of each plant with solvents of

increasing polarity gave 12 extracts. The ethanol extracts gave yields of 18%, 14%, and 22% (w/w, from 5 kg of dried leaves) for *B. pinnatum*, *C. officinalis*, and *A. officinalis*, respectively. The phytochemical screening results are shown in Table 1.

3.1 Extraction yield and phytochemical screening

Preliminary phytochemical screening revealed considerable variation in secondary metabolite distribution among the extracts, reflecting the influence of solvent polarity on phytoconstituent extraction. Cardiac glycosides, steroids, and terpenoids were detected in all 12 extracts, indicating that these metabolite classes were consistently recovered regardless of extraction solvent or plant species. Phenolic compounds, carbohydrates, and flavonoids were detected in 9 of the 12 extracts, whereas saponins and tannins were detected in 8. Alkaloids were detected in 7 of the 12 extracts. Anthraquinones showed the most restricted distribution and were detected only in the dichloromethane extract of *C. officinalis*.

A clear solvent-dependent pattern was observed. The *n*-hexane extracts contained only cardiac glycosides, steroids, and terpenoids, whereas the ethyl acetate and ethanol extracts of all three plants showed positive reactions for all the screened phytochemical classes except anthraquinones. Thus, the number and diversity of detectable phytoconstituents generally increased as extraction solvent polarity increased. This observation is consistent with solvents of different polarities preferentially dissolving compounds based on their physicochemical properties. The broader phytochemical profiles obtained with ethyl acetate and ethanol suggest that these solvents extracted a wider range of polar and moderately polar constituents than *n*-hexane. The occurrence of phenolics, flavonoids, tannins, saponins, and alkaloids in the more polar extracts is particularly relevant to the subsequent antimicrobial evaluation because these classes comprise chemically diverse compounds that have been associated with



biological activities in medicinal plants. However, the qualitative presence of a phytochemical class does not establish its concentration or demonstrate that it is responsible for the antimicrobial activity observed in the present study. The antimicrobial effects should therefore be interpreted as properties of the crude extracts rather than attributed to individual phytochemical classes without further isolation and mechanistic studies.

The relatively broad phytochemical profile of

the ethyl acetate and ethanol extracts provided a basis for their further evaluation and, in particular, for the GC–MS characterization of the ethanol extracts. The results demonstrate that solvent selection is an important consideration in developing plant-based antimicrobial materials because extraction conditions can substantially influence the chemical composition and, consequently, the biological properties of the resulting extracts.

Table 1. Phytochemical screening of sequential extracts of *A. officinalis* (AO), *B. pinnatum* (BP) and *C. officinalis* (CO)

Constituent	Test	AO H	AO D	AOE t	AO E	BP H	BP D	BPE t	BP E	CO H	CO D	COE t	CO E
Alkaloids	Dragendorff	–	–	+	+	–	+	+	+	–	–	+	+
Cardiac glycosides	Keller–Kiliani	+	+	+	+	+	+	+	+	+	+	+	+
Saponins	Frothing	–	–	+	+	–	+	+	+	–	+	+	+
Phenolics	Lead acetate	–	+	+	+	–	+	+	+	–	+	+	+
Tannins	Ferric chloride	–	–	+	+	–	+	+	+	–	+	+	+
Steroids	Salkowski	+	+	+	+	+	+	+	+	+	+	+	+
Carbohydrates	Molisch	–	+	+	+	–	+	+	+	–	+	+	+
Flavonoids	Shinoda	–	+	+	+	–	+	+	+	–	+	+	+
Terpenoids	Liebermann–Burchard	+	+	+	+	+	+	+	+	+	+	+	+
Anthraquinones	Borntrager	–	–	–	–	–	–	–	–	–	+	–	–

****H, n-hexane; D, dichloromethane; Et, ethyl acetate; E, ethanol extract. (+) present; (–) absent.**

3.1.2 GC–MS profile of the ethanol extracts

The major constituents identified in the ethanol extracts by GC–MS are presented in Table 2. The chromatographic profiles demonstrated clear differences in the relative abundance and distribution of compounds among the three plant species. Nevertheless, fatty acid-derived compounds were prominent in the extracts, particularly in *C. officinalis* and *B. pinnatum*. These

compositional differences indicate that the three plants provide distinct chemical profiles despite being extracted under the same solvent conditions.

The *C. officinalis* extract showed the strongest dominance of fatty acid methyl esters. Oleic acid methyl ester was the major detected constituent, accounting for 42.32% of the chromatographic profile, followed by methyl palmitate at 27.80%. Other constituents included linoleic acid methyl



ester, methyl stearate, and several long-chain fatty acid esters, while squalene represented 0.67% of the detected composition. The high relative abundance of oleic acid methyl ester and methyl palmitate indicates that lipid-derived constituents constituted a substantial proportion of the compounds detected in the ethanol extract of *C. officinalis*.

The ethanol extract of *B. pinnatum* also contained oleic acid methyl ester as its most abundant identified constituent, although at a lower relative abundance of 20.80%. Other

notable constituents included octadecenoic acid methyl ester (7.21%), an oleic acid glycerol derivative (6.19%), and free oleic acid (4.79%). The occurrence of both esterified and free fatty-acid-related compounds suggests a chemically diverse lipid-associated fraction within the ethanol extract. The lower relative abundance of the major constituent compared with *C. officinalis* also indicates that the chemical profile of *B. pinnatum* was less dominated by a single compound.

Table 2. Major constituents of the ethanol extracts identified by GC-MS

Extract	Compound	RT (min)	Area (%)
<i>B. pinnatum</i>	Oleic acid methyl ester	—	20.80
	Octadecenoic acid methyl ester	—	7.21
	Oleic acid glycerol derivative	—	6.19
	Oleic acid	—	4.79
<i>C. officinalis</i>	Oleic acid methyl ester	13.468	42.32
	Methyl palmitate	11.378	27.80
	Linoleic acid methyl ester	13.376	5.29
	Methyl stearate	13.779	4.41
	13-Docosenoic acid methyl ester	18.922	2.86
	11-Eicosenoic acid methyl ester	16.132	2.58
	Docosahexaenoic acid methyl ester	18.316	1.74
	Squalene	23.518	0.67
<i>A. officinalis</i>	Eicosane	—	8.11
	Hexadecanoic acid methyl ester	—	4.80
	9-Octadecenal	—	4.56
	11-Octadecenoic acid methyl ester	—	4.54
	Octadecane	—	4.29
	Squalene	—	2.13

In contrast, the ethanol extract of *A. officinalis* displayed a more evenly distributed GC–MS profile. Eicosane was the most abundant identified compound, accounting for 8.11%, followed by hexadecanoic acid methyl ester (4.80%), 9-octadecenal (4.56%), 11-octadecenoic acid methyl ester (4.54%), octadecane (4.29%),

and squalene (2.13%). The relatively lower abundance of individual compounds compared with the dominant constituents of *C. officinalis* suggests a more heterogeneous distribution of detectable compounds in the *A. officinalis* extract.

The differences among the three GC–MS profiles may contribute to the differences



observed in their antimicrobial performances. However, the present GC–MS data establish chemical associations rather than direct causal relationships between individual compounds and antimicrobial activity. For example, the high relative abundance of oleic acid methyl ester in *C. officinalis* coincides with the strong antimicrobial activity subsequently observed for this extract, but this relationship alone does not demonstrate that oleic acid methyl ester is responsible for the activity. The antimicrobial response may instead result from the combined effects of several constituents, including compounds present at lower concentrations that may act additively or synergistically.

Identifying fatty acid derivatives, hydrocarbons, aldehydes, and terpenoid-related constituents is nevertheless important for understanding the chemical basis of the extracts used in the leather formulation. In particular, the substantial representation of fatty acid methyl esters in *C. officinalis* and *B. pinnatum* provides a useful chemical marker for differentiating their ethanol extracts. Further work involving chromatographic fractionation, purification of individual constituents, and bioassay-guided testing would be required to determine which compounds or combinations of compounds contribute most strongly to the antimicrobial effects.

Overall, the phytochemical and GC–MS findings demonstrate that the three medicinal plants possess chemically distinct but overlapping profiles. The broad phytochemical diversity observed during preliminary screening, together with the distinct compound distributions identified by GC–MS, supports their investigation as complementary components of a polyherbal antimicrobial formulation.

3.1.3 Antimicrobial activity of the extracand extract-loaded leather

Table 3 summarises the antimicrobial activity of the extracts and of VTL loaded with each extract. All three extracts were active against every test organism, with zones of inhibition (ZOI) of 18–42 mm. *C. officinalis* gave the

largest zone in the study, 42 mm against *S. aureus*, with the lowest MIC recorded (0.10 mg/mL). This zone was 1.5 times that of ciprofloxacin (28 mm). *B. pinnatum* was the most active extract against *E. coli* (37 mm; MIC 0.20 mg/mL), again exceeding ciprofloxacin (30 mm). Against *S. pneumoniae*, *C. officinalis* and *A. officinalis* (29 mm each) were more active than ciprofloxacin (21 mm), while *B. pinnatum* was the weakest (18 mm; MIC 1.25 mg/mL). *P. aeruginosa* was the least susceptible organism: its zones (24–28 mm) were all smaller than those of ciprofloxacin (40 mm), and its MICs were 0.50–0.78 mg/mL.

All three extracts showed strong anticandidal activity. Zones against *C. albicans* were 33–37 mm, compared with 20 mm for fluconazole, and MICs were 0.20–0.25 mg/mL. For every extract–organism combination, the MBC or MFC was exactly twice the MIC (MBC/MIC = 2), which shows the extracts were bactericidal and fungicidal rather than static.

Once incorporated into leather, the ZOI of the loaded material (ZOI-EL) ranged from 9 to 28 mm. The ratio ZOI-EL/ZOI, taken as the fraction of activity retained, averaged 76.8% over the 15 extract–organism pairs (Table 3). By extract, the means were 85.6% for *B. pinnatum*, 75.7% for *C. officinalis* and 69.0% for *A. officinalis*. Retention was complete for *C. officinalis* against *E. coli* and *A. officinalis* against *S. aureus* (100%), and lowest for *A. officinalis* against *S. pneumoniae* (31.0%). One pair was anomalous: *B. pinnatum*-loaded leather gave a larger zone against *S. pneumoniae* than the free extract (25 vs 18 mm; 138.9%). Against *C. albicans* every loaded leather gave a 19 mm zone, close to fluconazole (20 mm). Against *S. pneumoniae*, leather loaded with *B. pinnatum* (25 mm) or *C. officinalis* (24 mm) exceeded ciprofloxacin (21 mm).

3.1.4 FTIR evidence of extract incorporation into leather

FTIR spectra of each free extract, the corresponding extract-loaded leather and untreated VTL were superimposed to identify



band shifts and intensity changes. For the *A. officinalis* ethanol extract, the O–H stretching band moved from 3360 to 3297 cm^{-1} and broadened after incorporation, merging with the O–H/N–H band of the leather. The carbonyl band moved from 1709 to 1638 cm^{-1} , into the collagen amide I region, and became the strongest band in the spectrum.

The aromatic C=C band at 1507.7 cm^{-1} was reduced to a weak shoulder near 1545 cm^{-1} , and the C–O stretching band near 1030 cm^{-1} was more intense in the loaded sample than in either starting material.

Table 3. Antimicrobial activity of the plant extracts and extract-loaded vegetable-tanned leather

Extract	Organism	ZOI (mm)	ZOI-EL (mm)	Retention (%)	CIP (mm)	FLU (mm)	MIC (mg/mL)	MBC/MFC (mg/mL)
<i>B. pinnatum</i>	<i>S. aureus</i>	33	24	72.7	28	—	0.25	0.50
	<i>E. coli</i>	37	28	75.7	30	—	0.20	0.40
	<i>P. aeruginosa</i>	28	25	89.3	40	—	0.50	1.00
	<i>S. pneumoniae</i>	18	25	138.9	21	—	1.25	2.50
	<i>C. albicans</i>	37	19	51.4	—	20	0.20	0.40
<i>C. officinalis</i>	<i>S. aureus</i>	42	27	64.3	28	—	0.10	0.20
	<i>E. coli</i>	27	27	100.0	30	—	0.63	1.25
	<i>P. aeruginosa</i>	25	20	80.0	40	—	0.78	1.56
	<i>S. pneumoniae</i>	29	24	82.8	21	—	0.50	1.00
	<i>C. albicans</i>	37	19	51.4	—	20	0.20	0.40
<i>A. officinalis</i>	<i>S. aureus</i>	24	24	100.0	28	—	0.78	1.56
	<i>E. coli</i>	21	18	85.7	30	—	1.00	2.00
	<i>P. aeruginosa</i>	24	17	70.8	40	—	0.78	1.56
	<i>S. pneumoniae</i>	29	9	31.0	21	—	0.50	1.00
	<i>C. albicans</i>	33	19	57.6	—	20	0.25	0.50

****ZOI, zone of inhibition of the extract; ZOI-EL, zone of inhibition of extract-loaded leather; Retention = $\text{ZOI-EL}/\text{ZOI} \times 100$; CIP, ciprofloxacin; FLU, fluconazole; MIC, minimum inhibitory concentration; MBC/MFC, minimum bactericidal/fungicidal concentration.**

For the *B. pinnatum* ethanol extract, the strong O–H/C–H absorption of the free extract (62% T at 2922 cm^{-1}) weakened to 84–85% T after incorporation, close to the VTL baseline. The carbonyl band moved from 1637.7 cm^{-1} (about 76% T) in the free extract to 1628.8 cm^{-1} (69% T) in the loaded leather, lower in wavenumber and stronger than in VTL alone (1654.9 cm^{-1} , 74% T). The

C. officinalis extract behaved similarly: its sharp hydroxyl and carbonyl bands weakened and shifted toward the leather bands after incorporation, while the C–O region near 1030 cm^{-1} regained intensity.

Fig. 1 compares the spectrum of the polyherbal (combined) insole with that of VTL.



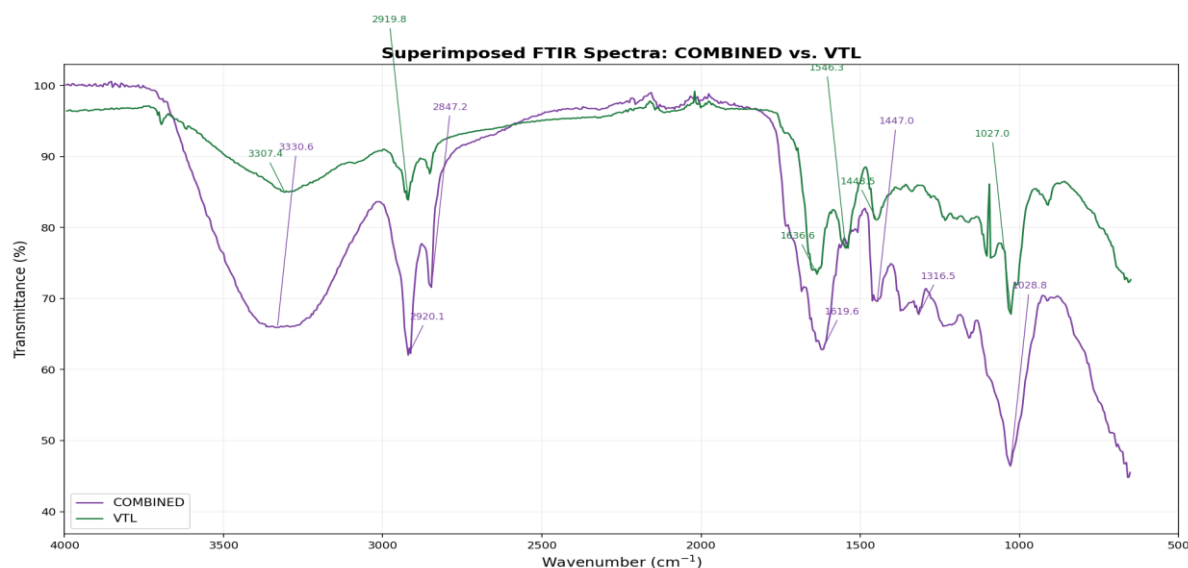


Fig. 1: Superimposed FTIR spectra of the polyherbal (combined *A. officinalis*, *B. pinnatum* and *C. officinalis*) extract-loaded insole and untreated vegetable-tanned leather (VTL)

Every major band of the combined insole absorbed more strongly than the corresponding VTL band: O–H stretch at 3330.6 cm^{-1} (66% T vs 85% T for VTL at 3307.4 cm^{-1}), C–H stretch at 2920.1 cm^{-1} (62% T vs 84% T), C=O/amide I at 1619.6 cm^{-1} (64% T vs 73% T at 1636.6 cm^{-1}) and C–O stretch at 1028.8 cm^{-1} (46% T vs 71% T at 1027.0 cm^{-1}). The bending region at $1440\text{--}1546\text{ cm}^{-1}$ changed little. The downward shift of the amide I band by about 17 cm^{-1} and the much stronger C–O and O–H absorptions show that the combined extract was taken up

in large amounts and interacts with the collagen matrix.

3.1.5 Stability and potency-decay kinetics of the polyherbal insole

Table 4 shows the antimicrobial potency of the polyherbal insole during use. The ZOI fell steadily from 42 mm on Day 0 to 16 mm on Day 84 and did not change between Day 84 and Day 96. Relative potency, taken as the ZOI at each time point divided by the Day 0 ZOI, remained above 90% up to Day 14 (90.5%), fell below 50% between Day 56 (57.1%) and Day 70 (47.6%), and levelled off at 38.1%.

Table 4: Zone of inhibition and relative potency of the polyherbal insole during storage

Storage time (days)	ZOI (mm)	Relative potency (%)	ln ZOI
0	42	100.0	3.738
14	38	90.5	3.638
28	35	83.3	3.555
42	28	66.7	3.332
56	24	57.1	3.178
70	20	47.6	2.996
84	16	38.1	2.773
96	16	38.1	2.773

****Relative potency = $\text{ZOI}_t / \text{ZOI}_0 \times 100$.**

The potency data were fitted to a first-order decay model, $\ln \text{ZOI}_t = \ln \text{ZOI}_0 - kt$. Using

only Day 0 and Day 84 gave $k = 0.0115\text{ day}^{-1}$, $t_{1/2} = 0.693/k = 60.3\text{ days}$ and $t_{90} = 0.105/k =$



9.1 days. Linear regression of $\ln$ ZOI on time across all seven points from Day 0 to Day 84 (Day 96 excluded because of the plateau) gave nearly the same values: $k = 0.0116 \text{ day}^{-1}$ ($R^2 = 0.981$), $t_{1/2} = 59.6$ days and $t_{90} = 9.0$ days (Table 5; Fig. 2b). A zero-order model fitted

the same interval slightly better ($R^2 = 0.993$; $k_0 = 0.319 \text{ mm day}^{-1}$), giving $t_{90} = 13.2$ days and $t_{1/2} = 65.9$ days. Linear interpolation of the observed data placed the time to 90% potency at about 15 days and the time to 50% potency at about 66 days.

Table 5: Kinetic parameters for loss of antimicrobial potency of the polyherbal insole

Model / method	Rate constant	R^2	$t_{1/2}$ (days)	t_{90} (days)
First-order, two-point (Day 0 and 84)	0.0115 day^{-1}	—	60.3	9.1
First-order, regression (Day 0–84, $n = 7$)	0.0116 day^{-1}	0.981	59.6	9.0
Zero-order, regression (Day 0–84, $n = 7$)	$0.319 \text{ mm day}^{-1}$	0.993	65.9	13.2
Observed (linear interpolation)	—	—	≈ 66	≈ 15

****First-order: $t_{1/2} = 0.693/k$; $t_{90} = 0.105/k$. Zero-order: $t_{1/2} = 0.5 \text{ ZOI}_0/k_0$; $t_{90} = 0.1 \text{ ZOI}_0/k_0$, with $\text{ZOI}_0 = 42 \text{ mm}$.**

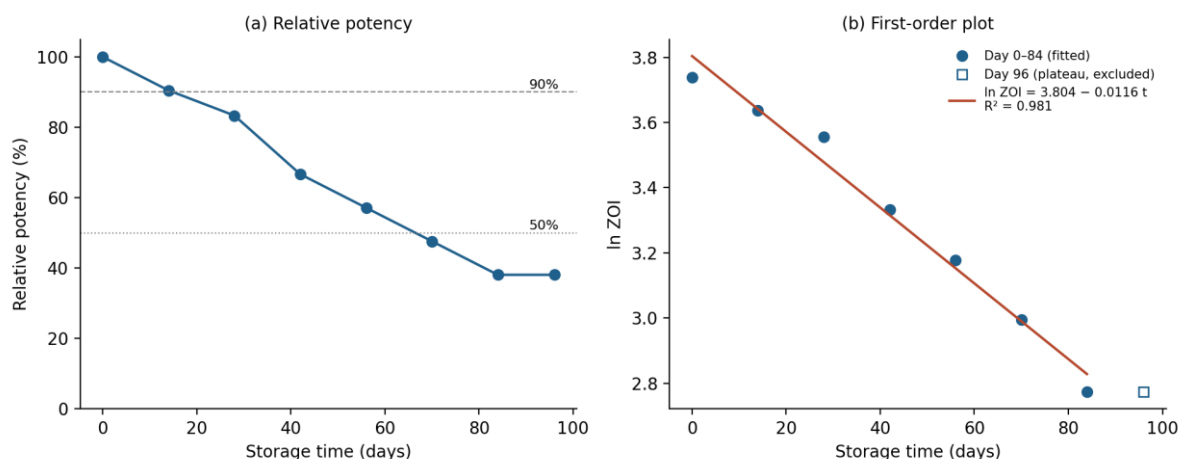


Fig. 2: Loss of antimicrobial potency of the polyherbal insole during storage. (a) Relative potency against time; dashed and dotted lines mark 90% and 50% potency. (b) First-order plot of $\ln$ ZOI against time, with linear regression over Days 0–84.

3.2 Discussion

Sequential extraction separated the phytochemicals of the three plants by polarity. Only lipophilic classes (steroids, terpenoids and cardiac glycosides) passed into n-hexane, whereas the ethyl acetate and ethanol fractions carried phenolics, flavonoids, tannins, saponins and alkaloids. This is the expected pattern for leaf material and matches earlier reports of flavonoids and triterpenes in *B. pinnatum* (Andrade *et al.*, 2020; Isibor, 2026), triterpenoid esters and flavonoids in *C. officinalis* (Shahane *et al.*, 2023) and phenolic acids, flavonoids and polysaccharides in *A. officinalis* (Bahari *et al.*, 2025). The same pattern is typical of

sequential extraction, in which non-polar solvents remove sterols and terpenoids and polar solvents take up glycosylated and phenolic constituents (Harborne, 1998; Evans, 2009). Phenolics, flavonoids and tannins can damage microbial membranes, bind proteins and chelate metals, and saponins make membranes more permeable (Scalbert, 1991; Cowan, 1999; Cushnie & Lamb, 2005; Daglia, 2012). Their presence in the polar fractions therefore helps explain the antimicrobial activity observed. The positive Molisch test in the polar *A. officinalis* fractions is consistent with its mucilage polysaccharides, which may help protect



tissue at the wound surface (Deters *et al.*, 2010; Bonaterra *et al.*, 2020).

GC-MS showed that fatty acid methyl esters, particularly derivatives of oleic acid, made up much of the volatile fraction of all three ethanol extracts. Long-chain unsaturated fatty acids and their esters disrupt bacterial membranes, uncouple the electron transport chain and inhibit fatty acid synthesis. Gram-positive bacteria such as *S. aureus* are generally more susceptible to them than Gram-negative bacteria (Desbois & Smith, 2010; Yoon *et al.*, 2018). Oleic and linoleic acids also modulate the inflammatory phase of wound healing and speed wound closure in animal models (Pereira *et al.*, 2008). The very high oleic acid methyl ester content of *C. officinalis* (42.3%) fits with its exceptional activity against *S. aureus* (42 mm; MIC 0.10 mg/mL). Conversely, the lower activity of all three extracts against *P. aeruginosa*, whose outer membrane and efflux systems limit the entry of hydrophobic molecules, fits the expected Gram-negative tolerance of fatty acids (Nikaido, 2003; Desbois & Smith, 2010). Squalene and long-chain alkanes such as eicosane in *A. officinalis* probably contribute antioxidant and barrier-supporting effects rather than strong antimicrobial action (Huang *et al.*, 2009). These associations are correlative, and bioassay-guided isolation of the active constituents is still needed.

All three extracts were active against every test organism, and several combinations were more active than the reference drugs. The most notable result was the anticandidal activity: all extracts produced zones 65–85% larger than fluconazole, with MICs of 0.20–0.25 mg/mL. This agrees with the antifungal activity reported for *Calendula* (Gazim *et al.*, 2008; Efstratiou *et al.*, 2012) and matters clinically, because fungal colonisation of chronic ulcers is common, is linked to delayed healing and is often overlooked (Kalan *et al.*, 2016). The MBC/MIC ratio of 2 across all combinations means the extracts kill organisms rather than merely inhibit them, which is desirable where immune clearance is impaired (Dawi *et al.*, 2025); an

MBC/MIC ratio of 4 or less is the usual criterion for cidal activity (Pankey & Sabath, 2004). Because each extract was strong against a different organism (*C. officinalis* against *S. aureus*, *B. pinnatum* against *E. coli*, and *C. officinalis*/*A. officinalis* against *S. pneumoniae*), combining them is a reasonable way to obtain broad-spectrum cover for the polymicrobial infections typical of DFUs (Senneville *et al.*, 2024). These findings agree with earlier reports of activity of *B. pinnatum* and *A. officinalis* leaf extracts against clinical bacterial isolates (Akinsulire *et al.*, 2007; Rezaei *et al.*, 2015). Comparisons with ciprofloxacin and fluconazole should nonetheless be read with care: crude extracts and pure drugs were tested at different concentrations, and zone size also depends on how well a substance diffuses through agar (Balouiri *et al.*, 2016). Incorporation into leather kept most of the antimicrobial activity (mean retention 77%), so the vegetable-tanned leather acted as a workable carrier rather than a sink. The partial losses are expected, because some extract is bound within the collagen matrix and cannot diffuse into the agar. The very low retention for *A. officinalis* against *S. pneumoniae* (31%) suggests that the constituents responsible for that activity are held especially tightly or are poorly released. The single retention above 100% (*B. pinnatum* against *S. pneumoniae*) may reflect synergy with residual vegetable tannins in the leather, which are themselves antimicrobial (Scalbert, 1991), or experimental variability. This result should be confirmed with replicate measurements and an untreated-leather control disc.

The FTIR data support true incorporation rather than a loose surface deposit. Red-shifted and broadened O–H bands, and movement of the extract carbonyl bands into the amide I region (about 1620–1640 cm⁻¹), are typical of hydrogen bonding between extract hydroxyl and carbonyl groups and the peptide backbone of collagen (Coates, 2000; Barth, 2007). Similar interactions underlie vegetable tanning itself, in which



polyphenols bind collagen through hydrogen bonds and hydrophobic interactions (Covington, 2009). The stronger C–O band near 1030 cm^{-1} is consistent with polysaccharide- and phenolic-rich material within the matrix. FTIR alone cannot show whether new covalent (for example, ester) bonds formed, and absorbance intensities in ATR-FTIR also depend on sample contact and loading. Spectroscopy that is sensitive to bonding (for example, XPS or solid-state NMR) together with release studies would help resolve this. The spectrum of the combined insole, in which every band absorbed more strongly than VTL, shows a high overall extract loading. This matches the large initial ZOI of the polyherbal insole (42 mm).

The stability study shows that the polyherbal insole loses antimicrobial potency steadily for the first 12 weeks. The first-order constant from regression (0.0116 day^{-1} ; $R^2 = 0.981$) agreed closely with the two-point estimate (0.0115 day^{-1}), so the half-life of about 60 days is robust. Several points affect how these results should be read. First, the diameter of an inhibition zone is roughly proportional to the logarithm of the diffusing agent's concentration, not the concentration itself (Bonev *et al.*, 2008). The ratio $\text{ZOI}_t/\text{ZOI}_0$ is therefore only an approximation of the fraction of active constituent remaining, and a standard curve of ZOI against log concentration would allow potency to be expressed in concentration terms. Second, the zero-order model fitted the Day 0–84 data slightly better than the first-order model ($R^2\ 0.993$ vs 0.981), so the data do not clearly distinguish between the two mechanisms. For a solid, matrix-bound system in which release or degradation happens at a surface, a zero-order or mixed process is plausible (Connors *et al.*, 1986). Third, the plateau at 16 mm between Day 84 and Day 96 suggests a residual fraction that is stable or firmly bound, possibly the extract held in the collagen matrix by hydrogen bonding as shown by FTIR, together with the

intrinsic activity of the vegetable-tanned leather.

The first-order t_{90} of about 9 days is short compared with the shelf lives usually expected of pharmaceutical products (ICH, 2003). However, observed potency was still 90.5% on Day 14, and interpolation of the measured data placed t_{90} at about 15 days, so the model-based value is conservative. In practice, the insole keeps more than half of its initial activity for about two months, and even at the plateau its zone (16 mm) remains within the range of useful activity. This is best treated as a design parameter rather than a shortcoming. It supports replacing the insole every two weeks, which fits with routine DFU review and footwear hygiene, or improving stability through protective packaging, storage in cool, dry and dark conditions, or adding an antioxidant or preservative to slow oxidation of the unsaturated fatty acid esters (Frankel, 2005). Accelerated stability testing at higher temperature and humidity, analysed with the Arrhenius equation, would allow shelf life to be predicted under real storage conditions (Waterman & Adami, 2005).

This study has limitations. We measured antimicrobial activity *in vitro* using agar diffusion, with a single time series for the insole. The authors did not assess cytotoxicity, skin sensitisation, or *in vivo* performance, and they followed potency for only about three months. Future work should include replicate stability studies at controlled temperatures and humidities, quantification of marker compounds during storage, cytotoxicity testing on keratinocytes and fibroblasts, evaluation in animal wound models and, eventually, controlled clinical trials in patients with DFUs.

4.0 Conclusion

Leaf extracts of *Bryophyllum pinnatum*, *Calendula officinalis* and *Althaea officinalis* are rich in phenolics, flavonoids, terpenoids and fatty acid esters, and all three have broad, cidal activity against bacterial and fungal pathogens isolated from diabetic foot ulcers. *C. officinalis* was the most potent against *S.*



aureus, and all three extracts outperformed fluconazole against *C. albicans*. The extracts were incorporated into vegetable-tanned leather through hydrogen-bonding interactions with collagen, and the loaded leather retained about three-quarters of the free-extract activity. The polyherbal insole lost potency with a half-life of about 60 days and a first-order t_{90} of about 9 days (about 15 days observed), which supports replacing the insole every two weeks or adding stabilising measures. Extract-loaded leather insoles are a promising, low-cost adjunct for preventing and managing infection in diabetic foot ulcers, subject to safety, *in vivo* and clinical evaluation.

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Compliance with Ethical Standards

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Ethical Approval

Not Applicable

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Authors' Contribution

The authors jointly contributed to the conceptualization, formulation, extraction, phytochemical screening, antimicrobial evaluation, FTIR characterization, and stability kinetics of the polyherbal leather insole. Akabuogu Ebere Promise coordinated the study and prepared the manuscript. Oyewale Adebayo Ojo, Habila James Dama, and Hamisu Ibrahim contributed to laboratory investigations, data analysis, interpretation of results, critical revision, and approval of the final manuscript.

