

Development of Phytochemical-Enriched Leather Insoles with Antimicrobial Efficacy for Potential Use in Diabetic Foot Ulcer Management

Akabuogu Ebere Promise*, Adebayo Ojo Oyewale, James Dama Habila, and Ibrahim Hamisu

Received: 28 June 2026/Accepted: 24 August 2026/Published: 12 September 2026

Diabetic foot ulcers (DFUs) are commonly associated with microbial infection and require approaches that can support infection control alongside appropriate footwear protection. This study developed phytochemical-enriched leather insoles using ethanolic extracts of *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis* and evaluated their phytochemical composition, antimicrobial activity, and chemical characteristics. Phytochemical screening detected nine metabolite classes, including alkaloids, cardiac glycosides, saponins, phenolics, tannins, steroids, carbohydrates, flavonoids, and terpenoids, while anthraquinones were absent. The crude extracts exhibited inhibition zones of 18–37 mm for *B. pinnatum*, 25–42 mm for *C. officinalis*, and 21–33 mm for *A. officinalis* against the tested microorganisms. *C. officinalis* produced the largest inhibition zone against *S. aureus* (42 mm), with MIC and MBC values of 0.10 and 0.20 mg/mL, respectively. *B. pinnatum* produced 37 mm inhibition zones against both *E. coli* and *C. albicans*, with corresponding MIC/MBC values of 0.20/0.40 mg/mL. Following incorporation into leather, inhibition zones ranged from 9–28 mm, with *B. pinnatum*-enriched leather producing 28 mm against *E. coli* and *C. officinalis*-enriched leather producing 27 mm against both *S. aureus* and *E. coli*. GC–MS analysis identified fatty acid derivatives as major constituents, including oleic acid methyl ester (42.32%) and methyl palmitate (27.80%) in *C. officinalis*, oleic acid methyl ester

(20.80%) in *B. pinnatum*, and eicosane (8.11%) in *A. officinalis*. FTIR analysis revealed characteristic O–H, C–H, and C–O/N–H absorption regions and spectral differences between untreated and extract-containing leather. The findings demonstrate the feasibility of producing antimicrobial phytochemical-enriched leather insoles, although further studies are required to establish their safety, durability, release characteristics, pressure-offloading performance, and clinical applicability in DFU management.

Keywords: Diabetic foot ulcer; Phytochemicals; Leather insoles; Antimicrobial activity; Plant extracts

Akabuogu Ebere Promise*

Directorate of Research and Development, Nigerian Institute of Leather and Science Technology (NILEST), Samaru P.M.B. 1034, Zaria, Nigeria

Email: beresunify16@gmail.com

Oyewale Adebayo Ojo

Department of Chemistry, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.

Email: oyewaleao@yahoo.co.uk

Habila James Dama

Department of Chemistry, Ahmadu Bello University, Zaria, Kaduna State, Nigeria

Email: habilajames2005@yahoo.com

Hamisu Ibrahim

Department of Chemistry, Ahmadu Bello University (ABU), Zaria, Nigeria

Email: hamisuibrahim@abu.edu.ng

1.0 Introduction

Diabetic foot ulcers (DFUs) are among the most debilitating complications of diabetes mellitus and represent a major challenge in wound care because of their chronicity, susceptibility to infection, delayed healing, and association with lower-limb amputation. The development and persistence of DFUs result from interconnected pathological processes that include peripheral neuropathy, impaired peripheral circulation, ischemia, excessive oxidative stress, persistent inflammation, impaired angiogenesis, altered immune responses, and repetitive mechanical loading of the plantar surface (Teixeira et al., 2023; Wang et al., 2025; Kandasamy & Alagarsamy, 2025). Once an ulcer develops, microbial colonization and infection can further disrupt the healing process and increase the risk of tissue destruction and systemic complications. Recent evidence indicates that chronic inflammation, oxidative imbalance, impaired tissue regeneration, and microbial biofilms are important factors underlying the poor healing response observed in diabetic wounds (Kasumbwe et al., 2026; Kandasamy & Alagarsamy, 2025). Thus, effective DFU care requires an integrated approach that addresses mechanical stress, infection, inflammation, and impaired tissue repair rather than relying exclusively on a single therapeutic intervention.

Mechanical loading is particularly important in the prevention and recurrence of diabetic foot ulcers. Neuropathy can reduce protective sensation, allowing repetitive plantar pressure and minor trauma to occur without adequate pain-mediated behavioral responses. Consequently, appropriate footwear and pressure-relieving devices constitute important components of DFU prevention and management. Lazzarini and Jarl (2021) emphasized the importance of off-loading interventions for reducing mechanical stress and facilitating ulcer healing, while a

systematic review by Suraya et al. (2025) identified evidence from eight eligible studies showing that appropriately designed insole footwear can reduce plantar pressure and the incidence of foot ulcers in patients with type 2 diabetes. Similarly, Ren et al. (2024) highlighted the importance of total-contact customization, compliant and absorbent materials, porous structures, and performance evaluation in the development of diabetic foot insoles. These findings establish the insole as an important component of diabetic foot-care technology. However, conventional pressure-relieving insoles primarily address mechanical loading and may not provide additional antimicrobial functionality.

Infection control remains another critical consideration in DFU management. Conventional approaches include antimicrobial agents, wound dressings, antibiotics, debridement, and off-loading interventions, but treatment can be complicated by antimicrobial resistance, persistent microbial colonization, biofilm formation, impaired host defense, and delayed wound healing (Teixeira et al., 2023; Wang et al., 2025). Recent evidence from a systematic review and network meta-analysis of 15 randomized controlled trials involving 1,292 patients indicates that antimicrobial dressings can influence DFU healing outcomes, although the effectiveness varies according to the antimicrobial technology and clinical context (He et al., 2026). The development of antimicrobial materials that can complement mechanical protection is therefore of considerable interest. Rather than viewing antimicrobial functionality and pressure off-loading as independent strategies, integrating biological activity into footwear materials may provide an opportunity to develop multifunctional products for diabetic foot care.

Natural products and plant-derived phytochemicals have attracted increasing attention as potential complementary



approaches to diabetic wound management. Phytochemicals possess diverse biological activities, including antimicrobial, antioxidant, anti-inflammatory, and pro-angiogenic effects, which are relevant to several pathological processes involved in diabetic wound healing (Kasumbwe et al., 2026; Kandasamy & Alagarsamy, 2025). Phytochemical classes such as flavonoids, phenolics, alkaloids, terpenoids, tannins, and saponins have been investigated for their ability to modulate oxidative stress, inflammatory responses, microbial growth, fibroblast proliferation, collagen production, extracellular-matrix remodeling, and angiogenesis (Kandasamy & Alagarsamy, 2025). Recent reviews further indicate that natural bioactive compounds are being incorporated into hydrogels, nanoparticles, electrospun nanofibres, polymeric scaffolds, and other delivery systems to improve stability, localization, and sustained release (Kasumbwe et al., 2026; Kandasamy & Alagarsamy, 2025). However, challenges involving formulation, standardization, stability, dose optimization, delivery, and clinical validation remain important barriers to their translation into routine DFU care (Kasumbwe et al., 2026).

The potential of naturally derived materials is also relevant to routine diabetic foot care. Krawiec et al. (2023) reviewed active ingredients and natural raw materials used in diabetic foot care and emphasized the importance of infection control, pressure relief, appropriate product formulation, skin protection, and microbiome management. Their review also identified plant extracts and other natural materials as potentially useful components of products intended for diabetic foot care. These observations support the broader concept of combining natural bioactive substances with materials designed for contact with the foot. Nevertheless, the incorporation of medicinal plant extracts into footwear materials presents additional technological

considerations, including extract–matrix interactions, retention of bioactive compounds, chemical compatibility, durability, and preservation of antimicrobial activity after incorporation.

Among medicinal plants investigated for antimicrobial and wound-related properties, *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis* are of particular interest. *Bryophyllum pinnatum* has demonstrated antimicrobial activity against selected clinical isolates, providing evidence of its potential as a source of antimicrobial phytochemicals (Ibikunle et al., 2017). More recent phytochemical screening of *B. pinnatum* leaf extract has also confirmed the presence of bioactive constituents and reported antimicrobial activity (Suryawanshi et al., 2025). *Calendula officinalis* is similarly recognized for its diverse phytochemical constituents and pharmacological activities, including antimicrobial and other biological effects relevant to wound care (Dhingra et al., 2022). Studies investigating *C. officinalis* flower extracts have reported antimicrobial activity against bacterial organisms, further supporting its potential application as a natural antimicrobial source (Afanasyeva et al., 2016; Hamad et al., 2017). In the case of *Althaea officinalis*, Rezaei et al. (2015) reported antibacterial activity of the leaf extract and demonstrated wound-healing activity in an experimental excision-wound model. Collectively, these findings provide a scientific basis for investigating the three plants as sources of phytochemicals for incorporation into functional materials.

Leather presents an interesting substrate for such an application because of its flexibility, durability, breathability, and established use in footwear. Previous research has demonstrated the potential for plant-derived materials to contribute to leather-related technologies; for example, *Polygonum hydropiper* has been investigated for sustainable goatskin



preservation (Hossain et al., 2022). At the same time, research on multifunctional insoles has demonstrated that footwear materials can be engineered to provide functions beyond basic mechanical support. Liu et al. (2024), for example, developed a BaTiO₃-based composite insole with antibacterial properties and investigated its effects in relation to infected-wound healing. Such studies demonstrate the feasibility of incorporating functional properties into insole materials, while research on diabetic foot insoles has increasingly emphasized material selection, structural optimization, pressure redistribution, absorbency, customization, and performance assessment (Ren et al., 2024). However, the use of medicinal plant extracts as bioactive agents directly incorporated into leather insole matrices remains comparatively underexplored.

Despite growing evidence concerning the antimicrobial, anti-inflammatory, antioxidant, and wound-healing properties of phytochemicals, most phytochemical-based DFU research has focused on extracts or isolated compounds as topical preparations, pharmaceutical formulations, hydrogels, nanoparticles, or other drug-delivery systems (Kasumbwe et al., 2026; Kandasamy & Alagarsamy, 2025). Conversely, diabetic-foot-insole research has predominantly concentrated on plantar-pressure redistribution, structural design, material properties, customization, and mechanical performance (Suraya et al., 2025; Ren et al., 2024). Consequently, an important interdisciplinary gap remains between phytochemical-based antimicrobial strategies and functional diabetic-foot footwear technology. In particular, there is limited evidence on the incorporation of selected medicinal plant extracts into leather insole matrices, the chemical confirmation of extract incorporation, the characterization of their bioactive constituents, and the extent to which

antimicrobial activity is retained after integration into the leather substrate.

Addressing this gap is important because incorporation into a leather matrix could potentially transform a conventional footwear component into a multifunctional material capable of providing both physical and biological functions. Such a material could complement pressure-management strategies by providing localized antimicrobial activity at the footwear-foot interface. Nevertheless, antimicrobial activity of an extract-enriched insole should not be equated with clinical treatment of an established DFU. Clinical effectiveness would require additional evidence concerning pressure redistribution, extract release and bioavailability, cytocompatibility, skin safety, durability, moisture management, in vivo wound healing, and controlled clinical outcomes. The present study therefore represents an initial materials and antimicrobial investigation rather than a clinical evaluation of DFU treatment.

Against this background, this study aimed to develop phytochemical-enriched leather insoles using ethanolic extracts of *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis*. Specifically, the study characterized the phytochemical constituents of the plant extracts, identified major chemical constituents using gas chromatography–mass spectrometry (GC-MS), investigated the incorporation of the extracts into vegetable-tanned leather using Fourier-transform infrared (FTIR) spectroscopy, and evaluated the antimicrobial activity of both the crude extracts and extract-enriched insoles against selected microorganisms, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Candida albicans*.

The significance of the study lies in its integration of phytochemistry, antimicrobial science, and leather-material technology to develop a potentially multifunctional insole



material. By combining a footwear substrate with plant-derived bioactive compounds, the study provides a basis for investigating locally accessible and potentially cost-effective antimicrobial materials that could complement existing diabetic foot-care strategies. The findings may also contribute to future research on multifunctional diabetic footwear in which mechanical protection, moisture management, antimicrobial activity, and other therapeutic-support functions are incorporated into a single material platform.

2.0 Materials and Method

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, and voucher specimens were deposited under the numbers ABUH01894, ABUH09024, and ABUH01641 for *B. pinnatum*, *C. officinalis*, and *A. officinalis*, respectively.

The collected leaves were initially 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 then 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.

Representative specimens of the three medicinal plants used in the study are presented in Fig. 1.



Bryophyllum Pinnatum Plant



Althaea Officinalis Plant



Calendula Officinalis Plant

Fig. 1. Representative leaves of *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis* used for extract preparation

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. The use of solvents with different polarities was intended to facilitate the extraction of phytochemicals with varying physicochemical characteristics.

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 subsequently allowed to air-dry 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.

For the subsequent antimicrobial and leather-loading experiments, the ethanol extracts were used as the principal extracts. The extracts were subjected to preliminary phytochemical screening to establish the major classes of secondary metabolites present.

2.3 Preliminary Phytochemical Screening

The crude extracts obtained using ethanol, ethyl acetate, dichloromethane, and *n*-hexane were qualitatively screened for selected classes of secondary metabolites using established phytochemical tests. The tests included those for anthraquinones, tannins, saponins, flavonoids, cardiac glycosides, terpenoids, steroids, carbohydrates, phenolic compounds, and alkaloids.

2.3.1 Test for Anthraquinones

Anthraquinones were screened using Bornträger's test. Approximately 6 g of the powdered plant material was soaked in 10 mL of benzene for 10 min and subsequently filtered. Ten millilitres of 10% ammonia solution was added to the filtrate and the mixture was vigorously shaken for approximately 30 s. The development of a pink, violet, or red colour in the ammoniacal phase was interpreted as a positive indication for anthraquinones.

2.3.2 Test for Tannins

Tannins were assessed using bromine water and ferric chloride tests.

For the bromine water test, 10 mL of bromine water was added to approximately 0.5 g of the aqueous extract. Decolourization of the bromine water was taken as an indication of tannins.

For the ferric chloride test, approximately 0.5 g of the extract was mixed with 10 mL of distilled water and filtered. A few drops of 1% ferric chloride solution were added to 2 mL of the filtrate. The development of a blue-black, green, or blue-green colour was considered indicative of tannins.

2.3.3 Test for Saponins

Saponins were screened using the frothing test. Approximately 5 mL of distilled water was added to the aqueous crude extract in a test tube and the mixture was vigorously shaken. A few drops of olive oil were subsequently added and the mixture was shaken again. Persistent frothing or foam formation was considered evidence of saponins.

2.3.4 Test for Flavonoids

Flavonoids were screened using the Shinoda and alkaline reagent tests.

For the Shinoda test, magnesium ribbon and concentrated hydrochloric acid were added to the aqueous crude extract. The development of a pink or reddish colour after several minutes was interpreted as a positive reaction for flavonoids.

For the alkaline reagent test, 2 mL of 2% sodium hydroxide solution was added to the aqueous extract. The formation of an intense yellow colour that disappeared following the addition of dilute acid was considered indicative of flavonoids.

2.3.5 Test for Cardiac Glycosides

Cardiac glycosides were evaluated using the Keller–Kiliani test. A mixture containing 4 mL of glacial acetic acid and one drop of 2% ferric chloride solution was added to 10 mL of the aqueous plant extract. One millilitre of concentrated sulfuric acid was then carefully introduced. The formation of a brown ring at the interface between the two layers was considered indicative of cardiac glycosides.

2.3.6 Test for Terpenoids

Terpenoids were screened using the Liebermann reaction. Two millilitres each of acetic acid and chloroform were added to the aqueous crude extract. The mixture was cooled, after which concentrated sulfuric acid was carefully added. The development of a green colour was considered indicative of terpenoid or steroidal components.

2.3.7 Test for Steroids



Steroids were screened using the Salkowski test. Two millilitres of concentrated sulfuric acid were carefully added to the aqueous crude extract. The development of a reddish-brown colour was considered evidence of steroidal constituents.

2.3.8 Test for Carbohydrates

Carbohydrates were detected using Molisch's test. A few drops of Molisch's reagent were added to the extract solution, followed by the careful addition of concentrated sulfuric acid along the wall of the test tube to form a separate lower layer. The formation of a violet-coloured ring at the interface was considered a positive reaction for carbohydrates.

2.3.9 Test for Phenolic Compounds

Phenolic compounds were screened using the lead acetate test. A few drops of lead acetate solution were added to the extract solution. The formation of a yellow precipitate was interpreted as evidence of phenolic compounds.

2.3.10 Test for Alkaloids

Alkaloids were screened using Mayer's, Wagner's, and Dragendorff's reagents. A few drops of the respective reagent were added to the extract solution. The formation of a cream precipitate with Mayer's reagent, a whitish or brownish precipitate with Wagner's reagent, or an orange-coloured precipitate with Dragendorff's reagent was interpreted as a positive reaction for alkaloids.

2.4 Study Design

The study employed a laboratory-based *in vitro* experimental design involving the preparation and characterization of medicinal plant extracts and the evaluation of their antimicrobial activity before and after incorporation into vegetable-tanned leather. Clinical specimens were obtained from five patients diagnosed with diabetic foot ulcers (DFUs) and attending the Ahmadu Bello University Medical Centre (ABUTH Sickbay), Zaria, Kaduna State, Nigeria.

Microorganisms recovered from the DFU specimens were isolated and identified using standard microbiological procedures. The antimicrobial activities of the selected plant extracts and extract-incorporated leather materials were subsequently evaluated using agar diffusion assays, while minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for the extracts.

Appropriate positive and negative controls were included in the antimicrobial assays. The positive controls consisted of a standard antibacterial agent and an antifungal agent, while solvent-only preparations and untreated leather were used as negative controls, as appropriate for each assay.

2.5 Ethical Considerations and Informed Consent

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Ethical approval was obtained from the Human Ethics Committee of Ahmadu Bello University before commencement of clinical specimen collection.

Written informed consent was obtained from each participant before the collection of DFU specimens. Participation was voluntary, and all specimens were coded to maintain participant confidentiality.

2.6 Inclusion and Exclusion Criteria

Patients of either sex who had been clinically diagnosed with diabetes mellitus and presented with a diabetic foot ulcer were eligible for inclusion in the study, irrespective of age.

Patients with diabetes mellitus but without foot ulceration were excluded. Non-diabetic individuals presenting with open wound infections were also excluded. Participants who had not provided informed consent were not included in the study.

2.7 Collection of Diabetic Foot Ulcer Specimens

DFU specimens were collected using a standardized wound-sampling procedure



(Ramsay et al., 2016). Before specimen collection, the wound was appropriately cleaned and prepared. Necrotic tissue, foreign material, calluses, and undermined wound edges were removed by debridement where clinically indicated. No topical antimicrobial agents, including alcohol- or iodine-containing preparations, were applied immediately before specimen collection, thereby minimizing the possibility of suppressing viable microorganisms in the specimen (Camilleri Attard et al., 2024).

Following wound preparation, specimens were collected using the Levine quantitative swab technique (Levine et al., 1976). A sterile swab was rotated over an approximately 1 cm² area of the wound bed for approximately 5 s while applying sufficient pressure to express fluid from the wound tissue. The swabs were immediately placed into brain–heart infusion broth and transported to the Microbiology Laboratory, Department of Microbiology, Ahmadu Bello University, within 30 min for microbiological processing.

All clinical specimen collection procedures were performed under appropriate clinical supervision.

2.8 Preparation of Culture Media

Blood agar, nutrient agar, MacConkey agar, and mannitol salt agar (MSA) were prepared in accordance with the respective manufacturers' instructions. The prepared media were sterilized and allowed to cool before use. Sterility and performance checks were performed as appropriate before inoculation.

2.9 Isolation and Identification of Microorganisms

Clinical specimens were inoculated onto blood agar, nutrient agar, MacConkey agar, and mannitol salt agar using standard microbiological procedures. The inoculated plates were incubated at 37 °C for 24 h, except where organism-specific growth requirements necessitated otherwise.

Distinct colonies were examined based on colony morphology, pigmentation, haemolytic characteristics, and other observable features. Representative colonies were subcultured on appropriate media to obtain pure cultures. Pure isolates were maintained as stock cultures under appropriate storage conditions until further characterization.

MacConkey agar was used primarily for the isolation and differentiation of Gram-negative enteric bacteria, whereas MSA provided selective conditions for the recovery of staphylococci and differentiation based on mannitol fermentation. Blood agar was used to support the recovery of a broad range of clinically relevant bacteria.

2.10 Morphological and Biochemical Identification of Isolates

The recovered bacterial isolates were identified using a combination of colony morphology, Gram staining, cellular morphology, and conventional biochemical tests, following established microbiological procedures (Dimri et al., 2020). Gram-positive isolates were subjected to catalase, coagulase, and mannitol fermentation tests, as appropriate. Gram-negative isolates were evaluated using tests including lactose fermentation, indole, and oxidase reactions.

The identification and antimicrobial susceptibility testing procedures were conducted with reference to applicable Clinical and Laboratory Standards Institute (CLSI) recommendations (Clinical and Laboratory Standards Institute [CLSI], 2020, 2024; Humphries et al., 2019).

2.11 Preparation of Extract Solutions

The dried ethanolic extracts of *B. pinnatum*, *C. officinalis*, and *A. officinalis* were used for the antimicrobial assays. Stock solutions were prepared by dissolving 1.0 g of each dried extract in 10 mL of the appropriate solvent to obtain a nominal concentration of 100 mg/mL. The stock solutions were subsequently diluted to obtain working concentrations of 50, 25, and



12.5 mg/mL using the dilution procedure described by Mitra (2004).

All extract solutions were freshly prepared or stored under conditions appropriate for maintaining their stability before use.

2.12 Preparation and Phytochemical Loading of Leather Insoles

Vegetable-tanned leather sheets were used as the substrate for preparation of the experimental insoles. The leather was cut into insole templates corresponding to the desired footwear dimensions. Separate circular leather discs, approximately 5 cm in diameter, were prepared for antimicrobial susceptibility testing.

For extract loading, ethanolic plant extracts were prepared at concentrations ranging from 200 to 400 mg/mL in 10% ethanol. The leather discs and insole templates were separately immersed in the respective extract solutions

and maintained at room temperature for 24 h to facilitate penetration and adsorption of the phytochemical constituents into the leather matrix (Hossain et al., 2022). Where applicable, chitosan at concentrations of 0.5–2.0% (w/v) was incorporated into the extract formulation to enhance adhesion of the bioactive material to the leather substrate and potentially facilitate controlled release (Do et al., 2023).

After the loading period, the leather specimens were removed from the extract solutions and air-dried under sterile conditions. Untreated vegetable-tanned leather was used as the material control, whereas leather treated with 10% ethanol without plant extract served as the solvent control.

The extract-loaded leather samples and the finished insole are shown in Figs. 2 and 3, respectively.



Fig. 2. Vegetable-tanned leather after incorporation of *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis* extracts

2.13 Antimicrobial Susceptibility Testing

The antimicrobial activities of the selected ethanolic plant extracts and extract-loaded leather discs were evaluated using an agar disc-diffusion method based on applicable CLSI procedures (CLSI, 2024). The test organisms comprised *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus*

pneumoniae, and *Candida albicans* recovered from DFU specimens.

Standardized microbial suspensions equivalent to approximately 0.5 McFarland turbidity were prepared in sterile saline. For bacterial assays, the suspensions were uniformly inoculated onto the surface of appropriate Mueller–Hinton agar plates to obtain confluent growth. The



Fig. 3. Representative finished leather insole prepared from extract-enriched vegetable-tanned leather



extract-loaded leather discs were aseptically placed on the inoculated agar surfaces and allowed to remain at room temperature for approximately 1 h to facilitate initial diffusion before incubation.

Bacterial plates were incubated at 37 °C for 24 h. *Candida albicans* plates were incubated at 28–30 °C for approximately 48 h. For *S. pneumoniae*, culture conditions appropriate for the organism, including enriched medium and a suitable atmosphere where required, were maintained.

Following incubation, the diameter of each zone of inhibition (ZOI) surrounding the test material was measured in millimetres using a transparent ruler or calibrated measuring device. Measurements were recorded as the total diameter of the clear zone, including the diameter of the test disc. Appropriate positive and negative controls were included in each assay.

2.14 Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of the ethanolic plant extracts was determined using a broth dilution procedure. Serial concentrations of each extract were prepared in sterile nutrient broth. Equal volumes of the respective extract preparations and standardized microbial inocula were combined in sterile test tubes.

The inoculated tubes were incubated at 37 °C for 24 h under appropriate conditions for the test organism. Following incubation, the tubes were examined for visible turbidity. The MIC was defined as the lowest concentration of the extract at which no visible microbial growth was observed.

2.15 Determination of Minimum Bactericidal Concentration

The minimum bactericidal concentration (MBC) was determined from the MIC assay by subculturing aliquots from tubes showing no visible growth onto Mueller–Hinton agar. The inoculated plates were incubated under

appropriate conditions for the respective test organism.

After incubation, the plates were examined for microbial growth. The MBC was defined as the lowest concentration of the plant extract that produced no visible colony growth on the subculture plate.

2.16 Gas Chromatography–Mass Spectrometry Analysis

Gas chromatography–mass spectrometry (GC–MS) was used to characterize the volatile and semi-volatile phytochemical constituents present in the plant extracts. The analysis was performed using electron-ionization mass spectrometry with a carrier gas maintained at a constant flow rate of 1.50 mL/min. An injection volume of 2 µL was used, and individual analytical runs lasted approximately 50–55 min.

The resulting chromatograms and mass spectra were processed using the instrument's dedicated software. Tentative compound identification was based on comparison of the experimental mass spectra and fragmentation patterns with reference spectra contained in the NIST and Wiley spectral libraries, following established approaches for mass-spectral compound identification (Oladunmoye et al., 2019; Hufsky & Böcker, 2017). The relative abundance of each identified constituent was estimated from its peak area relative to the total integrated peak area of the chromatogram (Ausloos et al., 1999).

Only compounds meeting the predefined spectral-matching criteria were considered in the final identification. Where applicable, compound identifications should be reported as *tentatively identified* unless confirmed using authentic reference standards.

2.17 Fourier-Transform Infrared Spectroscopic Analysis

Fourier-transform infrared (FTIR) spectroscopy was employed to investigate the chemical characteristics of the leather substrate and to provide evidence of extract



incorporation into the leather matrix. Three extract-loaded leather samples were examined: AOE, representing *A. officinalis*-loaded leather; BPE, representing *B. pinnatum*-loaded leather; and COE, representing *C. officinalis*-loaded leather. Untreated vegetable-tanned leather (VTL) served as the control.

FTIR spectra were acquired directly from intact leather specimens using the attenuated total reflectance (ATR) technique on an Agilent FTIR spectrometer. Spectral measurements were recorded over the range of 4000–650 cm^{-1} and reported in transmittance mode.

The spectra of the extract-loaded leather samples were compared with that of untreated vegetable-tanned leather to identify changes in characteristic absorption bands associated with the functional groups of the leather substrate and incorporated plant extracts. Differences in peak position, intensity, or the appearance of additional characteristic bands were interpreted as evidence of interactions between the plant-derived constituents and the leather matrix.

2.18 Experimental Controls and Quality Assurance

Appropriate positive, negative, and material controls were incorporated throughout the antimicrobial experiments to improve the reliability and interpretability of the results. Untreated vegetable-tanned leather and solvent-treated leather were used to establish the intrinsic antimicrobial background of the substrate and solvent, respectively. Standard antimicrobial agents were used as positive controls for comparison with the activity of the plant extracts and extract-loaded leather.

All microbiological procedures were conducted aseptically using sterilized materials and equipment. Culture media were prepared according to manufacturers' specifications, and microbial cultures were handled under appropriate laboratory conditions. Measurements obtained during antimicrobial assays were recorded systematically, and the

same measurement procedure was applied across all experimental groups.

2.19 Data Analysis

The experimental results were organized and summarized using appropriate descriptive statistics. Antimicrobial activity was expressed primarily as the diameter of the zones of inhibition (mm), while MIC and MBC values were reported in mg/mL. GC-MS results were expressed as the relative percentage abundance of the identified constituents based on chromatographic peak areas.

Where replicate measurements were performed, results should be reported as mean $\pm$ standard deviation (SD). Appropriate statistical tests should be applied to determine differences among treatment groups where the experimental design includes sufficient independent replicates.

3.0 Results

3.1 Phytochemical Screening of Ethanolic Leaf Extracts

Qualitative phytochemical screening was initially performed to establish the major classes of secondary metabolites present in the ethanolic leaf extracts of *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis*. As shown in Table 1, all three extracts exhibited a broadly similar phytochemical profile. Alkaloids, cardiac glycosides, saponins, phenolic compounds, tannins, steroids, carbohydrates, flavonoids, and terpenoids were detected in the extracts, whereas anthraquinones were not detected.

The occurrence of multiple classes of secondary metabolites indicates that the three extracts contain a chemically diverse mixture of potentially bioactive constituents. In particular, flavonoids, phenolic compounds, tannins, saponins, alkaloids, and terpenoids have been associated with antimicrobial, antioxidant, anti-inflammatory, and wound-related biological activities. The detection of these constituents is therefore consistent with



previous reports describing the phytochemical and pharmacological properties of *C. officinalis* and the antimicrobial properties of *B. pinnatum* (Dhingra et al., 2022; Ibikunle et al., 2017; Suryawanshi et al., 2025). The observed phytochemical diversity also provides a plausible basis for the antimicrobial effects subsequently observed in the crude extracts.

Table 1. Phytochemical constituents of the ethanolic leaf extracts of *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis*

Phytochemical constituent	Result
Alkaloids	+
Cardiac glycosides	+
Saponins	+
Phenolic compounds	+
Tannins	+
Steroids	+
Carbohydrates	+
Flavonoids	+
Terpenoids	+
Anthraquinones	–

Note: + = detected; – = not detected.

Anthraquinones were absent from all three ethanolic extracts. This finding indicates that the antimicrobial activity observed in the present study cannot be attributed to anthraquinone-containing constituents and instead may result from the combined or synergistic effects of the detected phytochemical classes. However, qualitative phytochemical screening does not establish the concentration or individual contribution of any particular compound; therefore, the GC-MS results presented subsequently provide additional chemical information on the constituents of the extracts.

3.2 Antimicrobial Activity of the Crude Plant Extracts

The antimicrobial activities of the ethanolic extracts were evaluated against five

microorganisms isolated from diabetic foot-ulcer specimens. The results, expressed as zones of inhibition (ZOI), minimum inhibitory concentrations (MIC), and minimum bactericidal concentrations (MBC), are presented in Table 2. All three plant extracts inhibited the growth of the tested microorganisms, although the magnitude of inhibition varied according to both the plant extract and test organism.

Among the three extracts, *C. officinalis* produced the largest inhibition zone, measuring 42 mm against *S. aureus*, and exhibited the lowest MIC and MBC values for this organism (0.10 and 0.20 mg/mL, respectively). The same extract also inhibited *C. albicans* with a ZOI of 37 mm and an MIC of 0.20 mg/mL. Against *S. pneumoniae*, *C. officinalis* produced a ZOI of 29 mm, while its activity against *E. coli* and *P. aeruginosa* was 27 and 25 mm, respectively. These results demonstrate that the activity of the *C. officinalis* extract extended across both Gram-positive and Gram-negative bacteria as well as the fungal test organism.

The *B. pinnatum* extract also demonstrated substantial antimicrobial activity. Its largest ZOIs were observed against *E. coli* and *C. albicans*, both measuring 37 mm, followed by *S. aureus* at 33 mm and *P. aeruginosa* at 28 mm. The lowest MIC values for *B. pinnatum* were recorded against *E. coli* and *C. albicans* (0.20 mg/mL), with corresponding MBC values of 0.40 mg/mL. In contrast, *S. pneumoniae* was less susceptible, with a ZOI of 18 mm and MIC and MBC values of 1.25 and 2.50 mg/mL, respectively.

The *A. officinalis* extract exhibited comparatively lower activity against *S. aureus* and *E. coli*, producing ZOIs of 24 and 21 mm, respectively. Nevertheless, it produced a ZOI of 29 mm against *S. pneumoniae* and 33 mm against *C. albicans*. Its MIC values ranged from 0.25 to 1.00 mg/mL, while MBC values ranged from 0.50 to 2.00 mg/mL. Thus, although the magnitude of activity differed



among the extracts, all three exhibited measurable activity against the complete test panel.

Table 2: Antimicrobial activity of ethanolic extracts of *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis* against selected diabetic foot-ulcer-associated microorganisms

Plant extract	Test organism	ZOI (mm)	MIC (mg/mL)	MBC (mg/mL)
<i>Bryophyllum pinnatum</i>	<i>S. aureus</i>	33	0.25	0.50
	<i>E. coli</i>	37	0.20	0.40
	<i>P. aeruginosa</i>	28	0.50	1.00
	<i>S. pneumoniae</i>	18	1.25	2.50
	<i>C. albicans</i>	37	0.20	0.40
<i>Calendula officinalis</i>	<i>S. aureus</i>	42	0.10	0.20
	<i>E. coli</i>	27	0.63	1.25
	<i>P. aeruginosa</i>	25	0.78	1.56
	<i>S. pneumoniae</i>	29	0.50	1.00
	<i>C. albicans</i>	37	0.20	0.40
<i>Althaea officinalis</i>	<i>S. aureus</i>	24	0.78	1.56
	<i>E. coli</i>	21	1.00	2.00
	<i>P. aeruginosa</i>	24	0.78	1.56
	<i>S. pneumoniae</i>	29	0.50	1.00
	<i>C. albicans</i>	33	0.25	0.50

Note: ZOI = zone of inhibition; MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration. Values are reported as means of triplicate determinations.

A comparison of MIC and MBC values further demonstrates that *C. officinalis* generally exhibited the greatest antimicrobial potency, particularly against *S. aureus*. The relatively low MIC values recorded for *B. pinnatum* against *E. coli* and *C. albicans* also indicate appreciable activity against these organisms. The comparatively high MIC/MBC values observed for *S. pneumoniae* with *B. pinnatum* and for *E. coli* with *A. officinalis* indicate greater concentrations were required to inhibit and eliminate these organisms.

The broad antimicrobial activity observed in the present study is consistent with previous reports of antimicrobial activity in *B. pinnatum* and *C. officinalis* (Afanasyeva et al., 2016; Hamad et al., 2017; Ibikunle et al., 2017; Suryawanshi et al., 2025). The activity of *A. officinalis* is also compatible with previous experimental evidence demonstrating

antibacterial and wound-healing properties of its leaf extract (Rezaei et al., 2015). The differences in susceptibility among microorganisms may reflect differences in cell-envelope structure, membrane composition, permeability, efflux mechanisms, and the interaction of individual microbial species with the complex mixture of phytochemicals present in each extract.

Overall, the results demonstrate that the three ethanolic extracts possess broad-spectrum *in vitro* antimicrobial activity, with *C. officinalis* showing particularly strong activity against *S. aureus*. These findings provided the basis for the subsequent incorporation of the extracts into leather and evaluation of whether their antimicrobial activity was retained after immobilization within the insole matrix.

3.3 Antimicrobial Activity of Extract-Enriched Leather Insoles



Following the demonstration of antimicrobial activity by the crude extracts, the ethanolic extracts were incorporated into vegetable-tanned leather to determine whether the resulting insole material retained antimicrobial

functionality. The zones of inhibition produced by the extract-enriched leather discs, together with the corresponding positive and negative controls, are presented in Table 3.

Table 3. Zones of inhibition produced by extract-enriched leather insoles and antimicrobial controls against selected microorganisms

Insole extract	Test organism	ZOI of Solvent enriched insole (mm)	Ciprofloxacin (+ve) (mm)	Fluconazole (+ve) (mm)
<i>B. pinnatum</i>	<i>S. aureus</i>	24	—	28
	<i>E. coli</i>	28	—	30
	<i>P. aeruginosa</i>	25	—	40
	<i>S. pneumoniae</i>	25	—	21
	<i>C. albicans</i>	19	—	20
<i>C. officinalis</i>	<i>S. aureus</i>	27	—	28
	<i>E. coli</i>	27	—	30
	<i>P. aeruginosa</i>	20	—	40
	<i>S. pneumoniae</i>	24	—	21
	<i>C. albicans</i>	19	—	20
<i>A. officinalis</i>	<i>S. aureus</i>	24	—	28
	<i>E. coli</i>	18	—	30
	<i>P. aeruginosa</i>	17	—	40
	<i>S. pneumoniae</i>	9	—	21
	<i>C. albicans</i>	19	—	20

Note: ZOI = zone of inhibition. Ciprofloxacin was used as the positive antibacterial control and fluconazole as the positive antifungal control. The negative control consisted of leather treated with solvent without plant extract. “—” indicates that the control was not applicable to that organism.

All three extract-enriched leather formulations retained measurable antimicrobial activity against the majority of the test organisms. The *B. pinnatum*-enriched leather produced ZOIs ranging from 19 to 28 mm, with the greatest inhibition against *E. coli* (28 mm), followed by

P. aeruginosa (25 mm), *S. pneumoniae* (25 mm), and *S. aureus* (24 mm). The inhibition zone against *C. albicans* was 19 mm. The *C. officinalis*-enriched leather exhibited ZOIs of 20–27 mm against the bacterial organisms and 19 mm against *C. albicans*. The greatest



activity was observed against both *S. aureus* and *E. coli*, with ZOI of 27 mm. Against *P. aeruginosa* and *S. pneumoniae*, the corresponding inhibition zones were 20 and 24 mm, respectively. These results indicate that the *C. officinalis* extract retained appreciable antimicrobial activity following incorporation into the leather matrix.

The *A. officinalis*-enriched leather showed ZOI ranging from 9 to 24 mm. The greatest activity was recorded against *S. aureus* (24 mm), followed by *E. coli* (18 mm) and *P. aeruginosa* (17 mm). The lowest inhibition was observed against *S. pneumoniae* (9 mm). Nevertheless, the formulation retained antifungal activity against *C. albicans*, producing a ZOI of 19 mm.

The solvent-only control produced no measurable inhibition, indicating that the observed antimicrobial activity was attributable to the incorporated plant extracts rather than the solvent treatment or leather substrate. The antibacterial positive control, ciprofloxacin, produced inhibition zones of 28–40 mm, while fluconazole produced a 20 mm zone against *C. albicans*. The enriched leather formulations therefore exhibited measurable activity relative to the standard antimicrobial controls, although the magnitude of inhibition varied according to the plant extract and microorganism.

A comparison of the crude-extract and extract-enriched leather results reveals an overall reduction in ZOI following incorporation into the leather matrix. For example, the *B. pinnatum* extract produced a 37 mm ZOI against *E. coli*, whereas the corresponding enriched leather produced 28 mm. Similarly, *C. officinalis* decreased from 42 to 27 mm against *S. aureus*, while *A. officinalis* decreased from 29 to 9 mm against *S. pneumoniae*. This reduction is expected because the free extract is directly available for diffusion into the agar,

whereas incorporation into a relatively dense leather matrix can restrict the mobility and release of active constituents. The interaction of phytochemicals with collagenous components of the leather may also reduce the concentration available at the agar–leather interface.

Despite this attenuation, the retention of antimicrobial activity after incorporation is an important finding because it demonstrates that the phytochemical functionality was not completely lost during leather processing. In particular, all three enriched leather formulations produced measurable inhibition against *C. albicans*, with ZOI of 19 mm, suggesting retention of antifungal activity after incorporation. The broad activity observed against Gram-positive and Gram-negative bacteria and *C. albicans* supports the potential of the material as an antimicrobial footwear substrate, although the present *in vitro* findings do not establish clinical efficacy in diabetic foot-ulcer treatment.

Table 4 provides a direct comparison between the inhibition zones produced by the crude extracts and those produced by the corresponding extract-enriched leather formulations. This comparison allows the extent of antimicrobial activity retained following incorporation into the leather matrix to be visualized.

The magnitude of retention varied among the three plants and microorganisms, suggesting that the antimicrobial performance of the enriched leather is influenced by both the chemical composition of the extract and the interaction between its constituents and the leather matrix. To facilitate visual comparison of the antimicrobial profiles, Fig. 4 presents the ZOI, MIC, and MBC values obtained for the three crude extracts against the test organisms.



Table 4: Comparison of zones of inhibition produced by crude plant extracts and corresponding extract-enriched leather formulations against selected microorganisms

Extracts	Organism	ZOI (mm)	ZOI-EL (mm)	CIPRO (mm)	FLU (mm)	MIC (mg/mL)	MBC/MFC (mg/mL)
B. pinnatum	S. aureus	33	24	28	-	0.25	0.5
	E. Coli	37	28	30	-	0.2	0.4
	P.	28	25	40	-	0.5	1
	Aeruginosa						
	S. Pneumoniae	18	25	21	-	1.25	2.5
	C. Albicans	37	19	-	20	0.2	0.4
C. officinalis	S. Aureus	42	27	28	-	0.1	0.2
	E. Coli	27	27	30	-	0.63	1.25
	P.	25	20	40	-	0.78	1.56
	Aeruginosa						
	S. Pneumoniae	29	24	21	-	0.5	1
	C. Albicans	37	19	-	20	0.2	0.4
A. officinalis	S. Aureus	24	24	28	-	0.78	1.56
	E. Coli	21	18	30	-	1	2
	P.	24	17	40	-	0.78	1.56
	Aeruginosa						
	S. Pneumoniae	29	9	21	-	0.5	1
	C. Albicans	33	19	-	20	0.25	0.5

Table 3. Antimicrobial activity of crude plant extracts and phytochemical-enriched leather against selected microorganisms

Plant extract	Test organism	ZOI (mm)	ZOI-EL (mm)	CIPRO (mm)	FLU (mm)	MIC (mg/mL)	MBC/MFC (mg/mL)
B. pinnatum	<i>S. aureus</i>	33	24	28	—	0.25	0.50
	<i>E. coli</i>	37	28	30	—	0.20	0.40
	<i>P.</i>	28	25	40	—	0.50	1.00
	<i>aeruginosa</i>						
	<i>S. pneumoniae</i>	18	25	21	—	1.25	2.50
	<i>C. albicans</i>	37	19	—	20	0.20	0.40
C. officinalis	<i>S. aureus</i>	42	27	28	—	0.10	0.20
	<i>E. coli</i>	27	27	30	—	0.63	1.25
	<i>P.</i>	25	20	40	—	0.78	1.56
	<i>aeruginosa</i>						



<i>A. officinalis</i>	<i>S. pneumoniae</i>	29	24	21	–	0.50	1.00
	<i>C. albicans</i>	37	19	–	20	0.20	0.40
	<i>S. aureus</i>	24	24	28	–	0.78	1.56
	<i>E. coli</i>	21	18	30	–	1.00	2.00
	<i>P. aeruginosa</i>	24	17	40	–	0.78	1.56
	<i>S. pneumoniae</i>	29	9	21	–	0.50	1.00
	<i>C. albicans</i>	33	19	–	20	0.25	0.50

Note: ZOI = zone of inhibition produced by the crude extract; ZOI-EL = zone of inhibition produced by extract-enriched leather; CIPRO = ciprofloxacin; FLU = fluconazole; MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; MFC = minimum fungicidal concentration; “–” = not applicable/not tested.

The comparative results indicate that the transition from free extract to leather-bound extract generally resulted in reduced inhibition zones, but substantial activity was retained in most extract–organism

As illustrated in Fig. 4, *C. officinalis* generally produced the largest inhibition zones and lowest MIC/MBC values, particularly against *S. aureus*. *B. pinnatum* also showed pronounced activity against *E. coli* and *C. albicans*, whereas *A. officinalis* exhibited comparatively moderate activity across most organisms. The graphical representation reinforces the complementary information obtained from ZOI and MIC/MBC measurements: ZOI provides an indication of inhibition under agar-diffusion conditions, whereas MIC and MBC provide concentration-dependent measures of inhibitory and bactericidal/fungicidal activity.

The MIC and MBC profiles of the three extracts are presented separately in Fig. 5 to provide a clearer comparison of extract potency across the test microorganisms.

The data show that the lowest MIC and MBC values were generally associated with the strongest antimicrobial responses. For *C. officinalis*, the MIC/MBC combination of 0.10/0.20 mg/mL against *S. aureus* represents the lowest concentration range observed in the study. In contrast, *B. pinnatum* required 1.25 and 2.50 mg/mL to inhibit and eliminate *S. pneumoniae*, respectively, representing its least potent response among the tested organisms. Similarly, *A. officinalis* showed its highest MIC/MBC values against *E. coli* (1.00/2.00 mg/mL).

The relationship between ZOI and MIC/MBC should, however, be interpreted cautiously. Although larger inhibition zones generally coincided with lower MIC values in the present dataset, ZOI is also influenced by compound diffusion through agar and therefore cannot be treated as a direct quantitative measure of antimicrobial potency. The combined use of ZOI, MIC, and MBC therefore provides a more comprehensive assessment of the antimicrobial properties of the extracts.



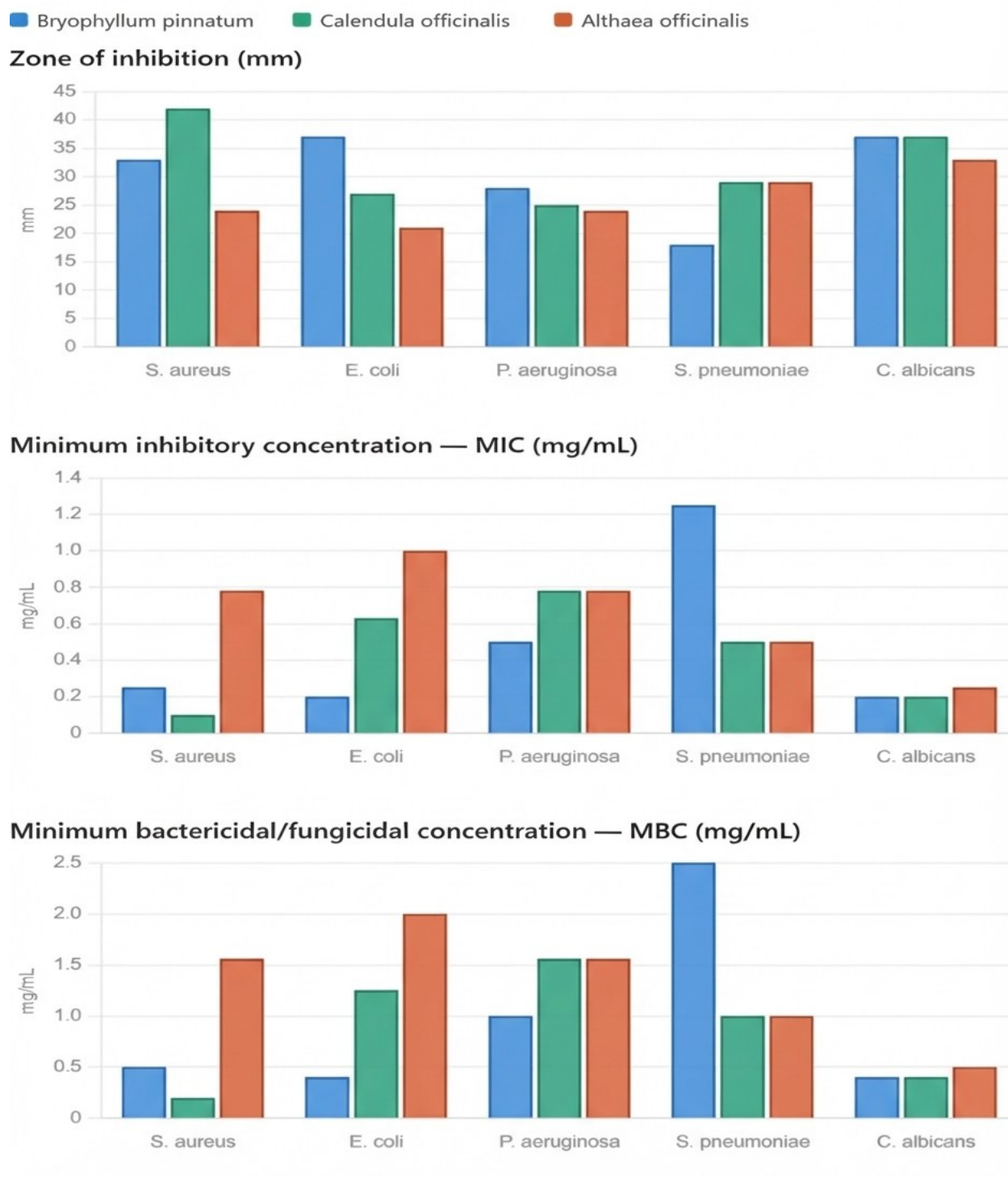


Fig. 4: Comparative antimicrobial profiles of the three ethanolic plant extracts based on zones of inhibition, minimum inhibitory concentrations, and minimum bactericidal concentrations



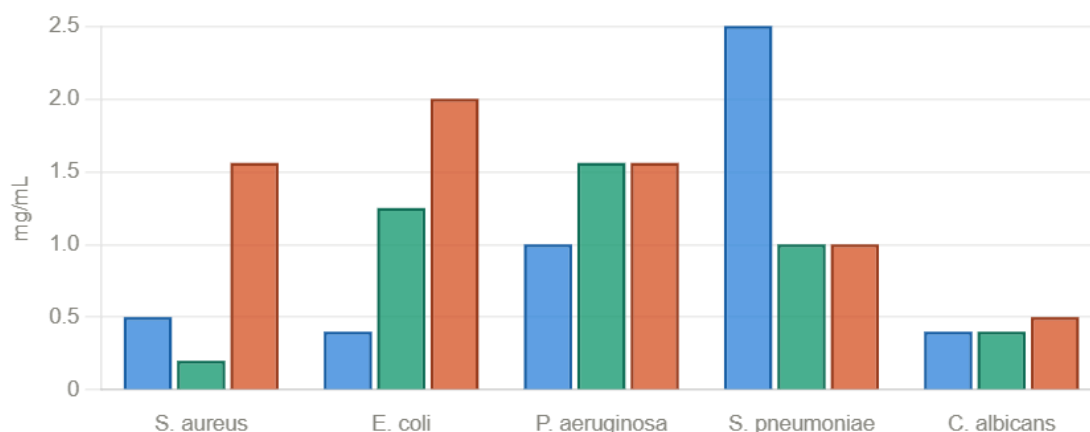


Fig. 5: Comparative minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) profiles of *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis* ethanolic extracts

The performance of the enriched leather formulations relative to the conventional antimicrobial controls is summarized graphically in Fig. 6.

Fig. 6 demonstrates that the extract-enriched leather formulations retained measurable antimicrobial activity against the test organisms, although the magnitude of inhibition was generally lower than that observed with the corresponding free extracts or standard antimicrobial agents. The absence

of inhibition in the solvent-treated leather controls further supports the conclusion that the observed activity originated from the incorporated plant extracts. Collectively, these findings demonstrate that incorporation into vegetable-tanned leather can preserve a substantial proportion of the antimicrobial functionality of the selected phytochemical extracts.

3

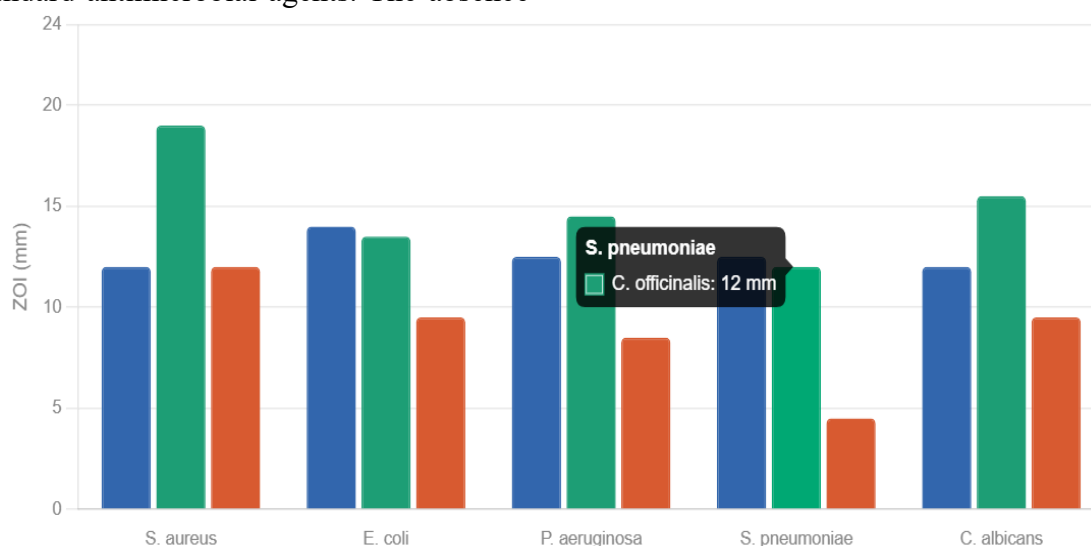


Fig 6: Zones of inhibition produced by extract-enriched leather insoles compared with the corresponding antibacterial and antifungal positive controls.



GC–MS analysis was performed to characterize the volatile and semi-volatile constituents present in the ethanolic extracts of *Calendula officinalis*, *Althaea officinalis*, and *Bryophyllum pinnatum*. The chromatographic profiles showed that the extracts contained predominantly fatty acid esters and other long-

chain lipid-related compounds, together with hydrocarbons, aldehydes, and minor bioactive constituents. The compounds reported below should be regarded as tentative identifications based on mass-spectral library matching, unless confirmed using authentic reference standards. The major compounds detected in the *C. officinalis* extract are presented in Table 5.

Retention time (min)	Tentatively identified compound	Relative abundance (%)
11.378	Methyl palmitate	27.80
13.376	Linoleic acid methyl ester	5.29
13.468	Oleic acid methyl ester	42.32
13.779	Methyl stearate	4.41
16.132	11-Eicosenoic acid methyl ester	2.58
18.316	Docosahexaenoic acid methyl ester	1.74
18.922	13-Docosenoic acid methyl ester	2.86
19.668	Bis(2-ethylhexyl) phthalate	1.76
23.518	Squalene	0.67

eicosenoic acid methyl ester (2.58%) were also detected, whereas docosahexaenoic acid methyl ester and squalene occurred at lower relative abundances of 1.74% and 0.67%, respectively. The chromatogram therefore indicates a profile dominated by long-chain fatty acid derivatives.

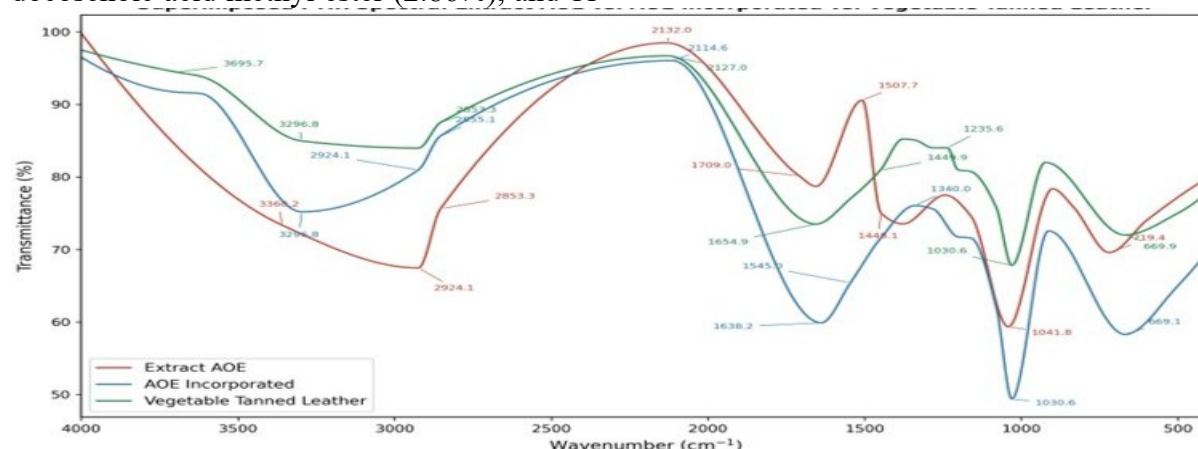


Fig. 7: Superimposed spectral extract ADE, AOE incorporated with VTL



Fig. 7 provides the chromatographic profile corresponding to the compounds summarized in Table 5. The prominent peaks associated with oleic acid methyl ester and methyl palmitate are consistent with their relatively high percentage contributions to the total detected chromatographic area. Bis(2-ethylhexyl) phthalate was also detected at 1.76%. Because phthalates may originate from laboratory plastics, tubing, solvents, or other

analytical materials, its occurrence should be treated cautiously and verified against procedural blanks before being considered a genuine phytochemical constituent.

The GC–MS profile of the *A. officinalis* extract is presented in Table 6. In contrast to *C. officinalis*, the profile contained appreciable amounts of long-chain hydrocarbons in addition to fatty acid derivatives and an aldehyde.

Table 6. Tentatively identified compounds in the *Althaea officinalis* extract by GC–MS

Retention time (min)	Tentatively identified compound	Relative abundance (%)
10.028	Octadecane	4.29
11.536	Hexadecanoic acid methyl ester	4.80
12.182	Eicosane	8.11
13.564	11-Octadecenoic acid methyl ester	4.54
14.411	9-Octadecenal	4.56
14.671	Carbonic acid hexadecyl prop-1-en-2-yl ester	5.39
17.433	3-Eicosene	3.46
23.526	Squalene	2.13

As indicated in Table 6, eicosane represented the largest single tentatively identified component in the *A. officinalis* extract, with a relative abundance of 8.11%. Other notable constituents included carbonic acid hexadecyl prop-1-en-2-yl ester (5.39%), hexadecanoic acid methyl ester (4.80%), 9-octadecenal

(4.56%), and 11-octadecenoic acid methyl ester (4.54%). Octadecane, 3-eicosene, and squalene accounted for 4.29%, 3.46%, and 2.13%, respectively. The occurrence of both saturated hydrocarbons and unsaturated lipid-derived compounds indicates a chemically diverse extract profile.

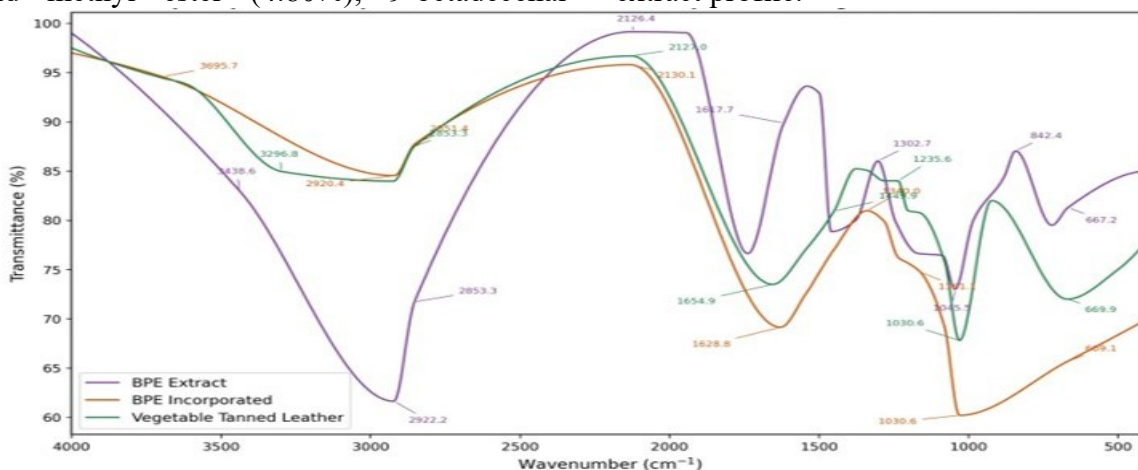


Fig. 8: superimposed spectra of BPE



The chromatogram in Fig. 8 demonstrates the distribution of the major peaks corresponding to the compounds listed in Table 6. The presence of squalene at 2.13% is noteworthy because this compound is a naturally occurring unsaturated hydrocarbon associated with lipid-rich biological matrices. However, the GC–MS result alone does not establish a specific

antimicrobial or wound-healing effect of the compound within the extract.

The GC–MS profile of *B. pinnatum* is presented in Table 7. The extract was characterized mainly by fatty acid esters and related lipid compounds, with oleic acid methyl ester and hexadecanoic acid methyl ester constituting the major detected components.

Table 7. Tentatively identified compounds in the *Bryophyllum pinnatum* extract by GC–MS

Retention time (min)	Tentatively identified compound	Relative abundance (%)
11.546	Hexadecanoic acid methyl ester	16.92
12.289	Hexadecanoic acid ethyl ester	8.47
13.567	Oleic acid methyl ester	20.80
13.877	Octadecenoic acid methyl ester	7.21
14.422	13-Octadecenal	9.83
14.746	Octadecanoic acid ethyl ester	5.59
27.163	Oleic acid glycerol derivative	6.19
27.790	Oleic acid	2.56
32.265	Oleic acid	2.23
33.316	Anthracene derivative/triterpene-like compound	4.35

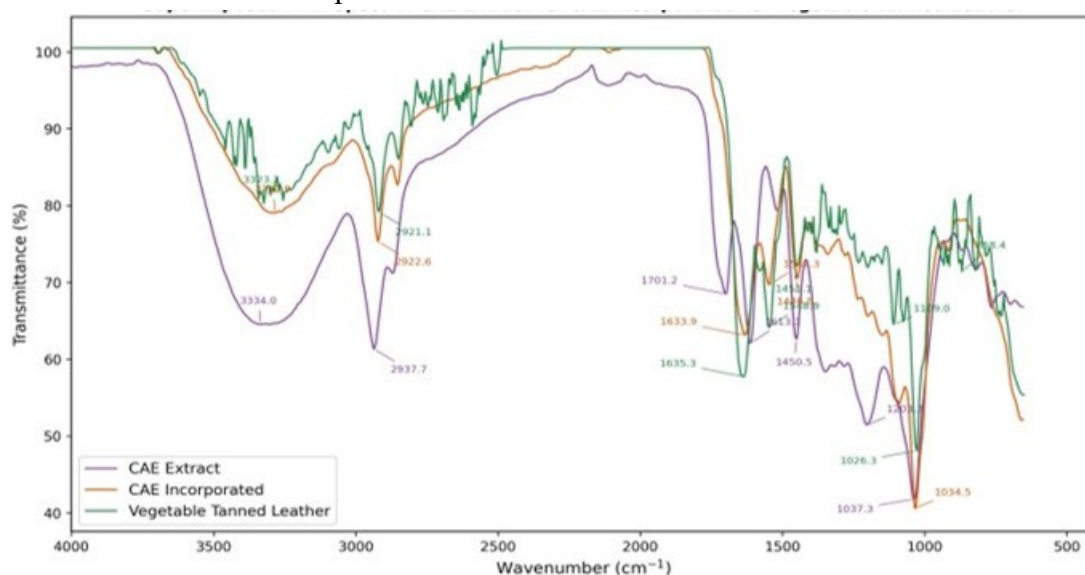


Fig. 9: superimposed spectra of CAE EXTRACT, CAE INCORPORATED and VTL

The results in Table 7 show that oleic acid methyl ester was the most abundant tentatively

identified compound in *B. pinnatum* (20.80%), followed by hexadecanoic acid methyl ester



(16.92%) and 13-octadecenal (9.83%). Hexadecanoic acid ethyl ester (8.47%), octadecenoic acid methyl ester (7.21%), the reported oleic acid glycerol derivative (6.19%), Fig. 9 illustrates the chromatographic pattern of the *B. pinnatum* extract. The relatively prominent peaks correspond to the fatty acid derivatives with the highest relative abundances in Table 7. Collectively, the GC–MS results for the three extracts demonstrate that fatty acid esters and related lipid constituents constitute important components of the analyzed extracts.

Although biological activities have been reported for several fatty acids and their derivatives, the present GC–MS analysis should not be interpreted as direct evidence that individual compounds were responsible for the antimicrobial activity observed in the agar diffusion assay. Rather, the chemical profiles provide a basis for relating the observed antimicrobial effects to the complex phytochemical composition of the extracts. In addition, the repeated identification of “oleic acid” at two different retention times in the *B. pinnatum* profile and the description of one

and octadecanoic acid ethyl ester (5.59%) were also detected. The profile therefore indicates substantial representation of fatty acid derivatives.

component as an “anthracene derivative/triterpene-like compound” warrant verification against the original mass spectra, library match scores, and retention indices before publication. Such verification would reduce the possibility of library misidentification.

3.5 FTIR Characterization of the Extracts and Leather Insole

FTIR spectroscopy was used to characterize the major functional groups present in the plant extracts and to examine changes in the chemical profile following incorporation of the extracts into the leather matrix. The spectra of *A. officinalis* extract (AOE), *B. pinnatum* extract (BPE), *C. officinalis* extract (COE), and the untreated leather sample (VTL) showed several common absorption regions, together with differences in band intensity and position. The comparative FTIR spectra are presented in Fig. 10.



Fig. 10: Combined extracts of AOE, BPE AND CAE Incorporated FTIR superimposed with VTL

As shown in Fig. 10, all four samples exhibited absorption features around 3695 and 3621

cm^{-1} , together with broad bands in the region of approximately 3296–3308 cm^{-1} . The high-



wavenumber bands are associated with O–H stretching vibrations, although the sharp bands around 3695 and 3621 cm^{-1} may also reflect hydroxyl-containing inorganic components if such materials are present in the samples. The broad bands around 3300 cm^{-1} are consistent with hydrogen-bonded O–H and/or N–H stretching vibrations.

Distinct absorption bands were also observed around 2920 and 2855 cm^{-1} in the analyzed samples. These bands are generally attributable to asymmetric and symmetric C–H stretching vibrations of aliphatic groups and are consistent with the presence of lipid-derived constituents identified by GC–MS. The strong absorption near 1030 cm^{-1} was another common feature of the spectra and may be associated with C–O stretching vibrations in oxygen-containing organic compounds. Assignment of this band specifically to Si–O–Si would require independent evidence for a silica-containing phase.

The AOE and BPE spectra exhibited broadly similar patterns, with additional absorption features in the region of approximately 913–915 cm^{-1} . The BPE spectrum also showed a relatively broad O–H region around 3235 cm^{-1} and a noticeable band near 1377 cm^{-1} . These differences indicate variations in the functional-group composition of the two extracts and may be related to differences in their constituent organic compounds.

The COE spectrum displayed an additional absorption feature near 874 cm^{-1} . This band may be associated with C–H deformation or, where carbonate-containing mineral components are present, carbonate-related vibrations. Therefore, assignment of the band specifically to carbonate minerals should be supported by complementary elemental or mineralogical evidence.

The untreated leather sample (VTL) exhibited a comparatively different spectral pattern. In particular, the band near 1030 cm^{-1} was less pronounced, while the feature around 913–915

cm^{-1} was diminished. An absorption band near 1654 cm^{-1} was also evident in the leather spectrum. This region is compatible with the amide I vibration of collagen, the principal structural protein in leather. The differences between the untreated leather and the plant-extract spectra demonstrate that the analyzed materials possessed distinguishable chemical profiles.

The comparative spectral changes observed among AOE, BPE, COE, and VTL provide spectroscopic evidence consistent with the presence of extract-derived functional groups in the analyzed formulations. However, FTIR alone cannot establish the extent of extract loading, molecular release, or covalent bonding between the extract constituents and the leather matrix. Quantitative loading and release studies would therefore be required to determine the amount and stability of phytochemicals retained within the leather during subsequent use.

4.0 Discussion

The present study investigated the development of phytochemical-enriched leather insoles incorporating ethanolic extracts of *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis*, with emphasis on their phytochemical composition, antimicrobial activity, chemical characteristics, and retention of antimicrobial activity following incorporation into leather. The findings demonstrate that all three plant extracts contained several classes of secondary metabolites and exhibited measurable antimicrobial activity against the tested bacterial and fungal organisms. Importantly, antimicrobial activity was retained after incorporation into the leather matrix, although the magnitude of inhibition generally decreased compared with the corresponding crude extracts. The GC–MS and FTIR results further indicated the presence of diverse organic constituents and functional groups that provide chemical evidence supporting the



composition of the extracts and their incorporation into the leather material.

4.1 Phytochemical Composition of the Plant Extracts

The preliminary phytochemical screening presented in Table 1 showed the presence of alkaloids, cardiac glycosides, saponins, phenolic compounds, tannins, steroids, carbohydrates, flavonoids, and terpenoids in the three plant extracts, whereas anthraquinones were not detected. The broad distribution of these phytochemical classes indicates that the plants contain chemically diverse constituents capable of contributing to biological activity. The detection of several of these metabolite classes is consistent with previous reports describing the phytochemical composition of *B. pinnatum* and *C. officinalis* extracts (Dhingra et al., 2022; Ibikunle et al., 2017; Suryawanshi et al., 2025).

The presence of phenolic compounds, flavonoids, tannins, terpenoids, and alkaloids is particularly relevant when interpreting the antimicrobial activity observed in the present study. These groups encompass compounds that have been associated in the literature with interactions with microbial membranes, proteins, enzymes, and other cellular components. However, the qualitative phytochemical screening used in this study establishes only the presence or absence of broad chemical classes and does not determine the concentration or identity of individual compounds. Consequently, the antimicrobial effects cannot be attributed to a particular phytochemical class on the basis of the screening results alone.

The presence of multiple phytochemical classes may nevertheless indicate the possibility of combined or complementary biological effects. For example, phenolic and flavonoid constituents may contribute to antimicrobial and antioxidant properties, while tannins and saponins have also been associated with antimicrobial effects. The broad

phytochemical profile observed in the present investigation therefore provides a plausible chemical basis for further investigation of these extracts as components of antimicrobial materials. The absence of anthraquinones in all three extracts further demonstrates that the phytochemical profiles were not identical to those of plants in which anthraquinone-containing constituents are prominent.

4.2 Antimicrobial Activity of the Crude Plant Extracts

The antimicrobial results in Table 2 demonstrated that all three extracts inhibited at least one of the tested microorganisms, although the magnitude of inhibition varied according to both the plant extract and test organism. *C. officinalis* produced the largest inhibition zone against *S. aureus* (42 mm), while *B. pinnatum* produced inhibition zones of 37 mm against both *E. coli* and *C. albicans*. *A. officinalis* also exhibited measurable activity, with inhibition zones ranging from 21 to 33 mm. These findings indicate that the extracts possessed broad-spectrum in vitro antimicrobial activity, although their activity profiles differed among microorganisms.

The particularly pronounced activity of *C. officinalis* against *S. aureus* was accompanied by the lowest MIC and MBC values recorded in the study, 0.10 and 0.20 mg/mL, respectively. This combination of a relatively large inhibition zone and low inhibitory and bactericidal concentrations suggests substantial in vitro activity against the tested *S. aureus* isolate. Previous studies have similarly reported antimicrobial activity of *C. officinalis* extracts against bacterial organisms, supporting the observation that this plant contains constituents capable of inhibiting microbial growth (Afanasyeva et al., 2016; Hamad et al., 2017; Jyotisree et al., 2020).

The *B. pinnatum* extract showed its strongest activity against *E. coli* and *C. albicans*, with inhibition zones of 37 mm and MIC/MBC values of 0.20/0.40 mg/mL. This observation is



consistent with previous reports of antimicrobial activity in *B. pinnatum* extracts against clinical bacterial isolates (Ibikunle et al., 2017; Suryawanshi et al., 2025). The activity against both a Gram-negative bacterium and a yeast suggests that the extract contains constituents capable of affecting organisms with substantially different cellular structures.

The *A. officinalis* extract produced inhibition zones of 24 mm against *S. aureus* and *P. aeruginosa*, 21 mm against *E. coli*, 29 mm against *S. pneumoniae*, and 33 mm against *C. albicans*. Its MIC values ranged from 0.25 to 1.00 mg/mL. These results demonstrate that the extract was active against both bacterial and fungal test organisms, although its activity was generally lower than that of the most active extract-organism combinations. The finding is compatible with previous experimental evidence reporting antibacterial activity and wound-healing effects of *A. officinalis* leaf extract (Rezaei et al., 2015).

Differences in susceptibility among the test organisms are expected because microbial cell-envelope structure, membrane composition, efflux mechanisms, enzymatic activity, and other physiological characteristics can influence the response to plant-derived compounds. Thus, the different inhibition zones should not be interpreted solely as differences in the intrinsic potency of the extracts. They also reflect differences in the susceptibility of the individual microorganisms.

It is important to distinguish the present in vitro findings from clinical antimicrobial efficacy. The observed inhibition zones demonstrate measurable antimicrobial activity under the experimental conditions used, but they do not by themselves establish clinical effectiveness against diabetic foot infections. Similarly, the MIC and MBC values obtained in vitro cannot be directly translated into therapeutic doses or

clinical treatment thresholds without additional pharmacological and clinical studies.

4.3 Antimicrobial Activity of the Phytochemical-Enriched Leather Insoles

The antimicrobial performance of the enriched leather insoles is presented in Table 3. Following incorporation into the leather matrix, all three plant formulations retained measurable activity against most of the organisms tested. The *B. pinnatum*-enriched leather produced inhibition zones of 24, 28, 25, 25, and 19 mm against *S. aureus*, *E. coli*, *P. aeruginosa*, *S. pneumoniae*, and *C. albicans*, respectively. The *C. officinalis*-enriched leather produced corresponding inhibition zones of 27, 27, 20, 24, and 19 mm, while the *A. officinalis*-enriched leather produced zones of 24, 18, 17, 9, and 19 mm.

The results demonstrate that antimicrobial activity was not completely lost during incorporation into the leather. This is an important observation because the usefulness of an antimicrobial footwear material depends not only on the antimicrobial properties of the active extract but also on the ability of the material to retain or release sufficient active constituents after formulation. The persistence of activity therefore provides preliminary evidence that leather can serve as a carrier matrix for these plant-derived extracts.

Comparison of Tables 2 and 3, however, shows that incorporation into leather generally reduced the inhibition zones relative to the corresponding crude extracts. For example, the inhibition zone of *C. officinalis* against *S. aureus* decreased from 42 mm for the crude extract to 27 mm following incorporation, whereas the *B. pinnatum* inhibition zone against *E. coli* decreased from 37 to 28 mm. The largest reduction was observed for *A. officinalis* against *S. pneumoniae*, where the inhibition zone decreased from 29 mm in the crude extract to 9 mm in the enriched leather. One possible explanation is that the leather matrix restricts diffusion of some extract



constituents through the agar. Compounds that are readily diffusible in an extract solution may diffuse more slowly after adsorption or retention within a relatively hydrophobic and structurally complex leather matrix. Similar considerations are relevant to other plant-extract-containing polymeric or biomaterial systems, where matrix interactions and controlled release can influence the amount of active compound available at the test interface. The observed reduction should therefore not necessarily be interpreted as chemical degradation of the extract.

The solvent control produced no measurable inhibition, indicating that the antimicrobial zones observed around the enriched leather were not attributable to the solvent treatment alone. The reference antibiotics also produced substantial inhibition against the bacterial isolates, while fluconazole produced a 20 mm zone against *C. albicans*. These controls provide an important basis for confirming that the assay was responsive under the experimental conditions.

Fig. 6 provides a visual comparison of the antimicrobial responses of the three enriched leather formulations with the reference antimicrobial controls. The Fig. illustrates the differences among the plant formulations and shows that the enriched leather materials retained activity while generally producing smaller zones than the corresponding reference antibiotics.

The retained activity is particularly relevant to the proposed application because a functional antimicrobial insole would ideally maintain antimicrobial properties while serving as a physical footwear component. Nevertheless, the present study did not evaluate the durability of antimicrobial activity following repeated flexing, washing, perspiration exposure, prolonged contact, or simulated walking. Such experiments are necessary before the practical performance of the material can be established.

4.4 GC–MS Profile and Its Relationship to Antimicrobial Activity

The GC–MS results presented in Tables 5–7 revealed substantial chemical differences among the three plant extracts, although fatty acid esters and other lipid-associated constituents were common features. *C. officinalis* was characterized particularly by oleic acid methyl ester (42.32%) and methyl palmitate (27.80%), whereas *A. officinalis* contained eicosane (8.11%), carbonic acid hexadecyl prop-1-en-2-yl ester (5.39%), and several fatty acid derivatives. In *B. pinnatum*, oleic acid methyl ester (20.80%), hexadecanoic acid methyl ester (16.92%), and 13-octadecenal (9.83%) were among the major tentatively identified constituents.

The dominance of fatty acid derivatives provides a chemical context for the antimicrobial activity observed in the agar diffusion experiments. Fatty acids and some related derivatives have been investigated for antimicrobial properties, and their activity has been associated in some systems with interactions with microbial membranes and alteration of membrane-associated processes. However, the present study did not isolate or individually test the compounds identified by GC–MS. Therefore, the GC–MS data should be interpreted as a chemical characterization of the extracts rather than direct evidence that any particular compound produced the observed antimicrobial response.

The comparatively high abundance of oleic acid methyl ester in *C. officinalis* is notable because this extract also demonstrated the strongest inhibition against *S. aureus*. Nevertheless, a direct causal relationship cannot be established from these observations. Antimicrobial activity in crude plant extracts commonly results from the combined effects of multiple constituents rather than from a single dominant GC–MS peak. Differences in compound polarity, concentration, synergistic



interactions, and diffusion behaviour may all influence the observed biological response. The presence of squalene in *C. officinalis*, *A. officinalis*, and *B. pinnatum* also demonstrates the occurrence of minor unsaturated hydrocarbon constituents. However, the relatively low abundance of squalene means that its contribution to the antimicrobial activity of the crude extracts cannot be inferred from the present data. Similarly, the detection of bis(2-ethylhexyl) phthalate in *C. officinalis* requires careful interpretation because phthalates are common environmental and laboratory contaminants. Confirmation using blanks and, ideally, authentic standards would strengthen the reliability of this identification. Overall, the GC–MS findings support the conclusion that the extracts contain chemically diverse constituents, including several lipid-related compounds that may contribute to their biological properties. Further fractionation, purification, and compound-specific antimicrobial assays would be required to establish the contribution of individual constituents.

4.5 FTIR Characteristics and Evidence of Extract Incorporation

The FTIR spectra in Fig. 10 demonstrated both common and distinct absorption features among the three extracts and untreated leather. The broad bands in the 3200–3300 cm^{-1} region are compatible with O–H and/or N–H stretching vibrations, while the bands around 2920 and 2855 cm^{-1} are consistent with aliphatic C–H stretching. These functional groups are expected in organic plant extracts containing fatty acids, alcohols, proteins, and other oxygenated compounds.

The strong absorption near 1030 cm^{-1} observed in the extracts may be associated with C–O stretching in oxygen-containing organic compounds. The precise assignment of this region, however, depends on the chemical composition of the samples and should not be

attributed to a single functional group without comparison with appropriate reference spectra. The BPE spectrum showed additional differences around 3235 and 1377 cm^{-1} , whereas COE exhibited a feature near 874 cm^{-1} . These spectral differences are consistent with variations in the chemical composition of the extracts. The untreated leather spectrum also differed from the extract spectra, particularly around 1030 and 913–915 cm^{-1} , and exhibited an absorption near 1654 cm^{-1} . The latter is compatible with the amide I region associated with the collagen structure of leather.

Taken together, the FTIR findings provide spectroscopic evidence consistent with differences between the untreated leather and extract-containing materials. These differences support the interpretation that plant-derived constituents were present in the formulated materials. However, FTIR cannot by itself demonstrate whether the extract constituents were covalently bonded to the leather, physically adsorbed, entrapped within the matrix, or simply deposited on the leather surface. Additional analyses, such as extraction/recovery studies, surface characterization, or quantitative release experiments, would be needed to determine the nature and stability of the interaction between the extracts and leather.

4.6 Integrated Assessment of the Phytochemical-Enriched Leather Insole

The combined findings from the phytochemical screening, antimicrobial assays, GC–MS, and FTIR analyses provide preliminary evidence for the feasibility of incorporating plant-derived extracts into leather to produce an antimicrobial footwear material. The three extracts displayed broad phytochemical profiles and measurable antimicrobial activity, while the enriched leather formulations retained antimicrobial activity against several of the tested microorganisms.



The significance of this approach lies in combining a conventional footwear material with biologically active plant-derived constituents. Diabetic foot ulcers are associated with multiple interacting factors, including mechanical stress, impaired healing, and susceptibility to microbial colonization and infection. Footwear and insole systems are important components of diabetic foot care because appropriate designs can reduce mechanical loading and pressure on vulnerable areas (Lazzarini & Jarl, 2021; Ren et al., 2024; Suraya et al., 2025). Separately, phytochemical-based approaches have attracted attention because plant-derived compounds may possess antimicrobial and other biological properties relevant to wound environments (Kasumbwe et al., 2026; Kandasamy & Alagarsamy, 2025).

The present material should therefore be viewed as a potential multifunctional footwear component rather than a demonstrated treatment for diabetic foot ulcers. The study established antimicrobial activity under laboratory conditions but did not measure plantar-pressure redistribution, off-loading performance, wound closure, tissue regeneration, cytotoxicity, or clinical outcomes. Consequently, the results support further development and testing rather than direct therapeutic application.

The retained antimicrobial activity after incorporation is nevertheless an important proof-of-concept finding. In practical applications, the performance of such a material would depend on the concentration of extract incorporated, release kinetics, stability during storage, resistance to mechanical wear, moisture and sweat exposure, compatibility with skin, and maintenance of activity during repeated use. These factors should be investigated before the material can be considered for clinical or commercial application.

4.7 Limitations and Future Research Directions

Several limitations should be considered when interpreting the findings. First, the antimicrobial experiments were conducted *in vitro* and therefore do not establish efficacy against diabetic foot ulcers in patients. The microbial isolates tested provide useful experimental evidence, but the number and diversity of isolates were limited and cannot represent the full microbial complexity of diabetic foot infections.

Second, the study did not evaluate the cytotoxicity or skin compatibility of the plant-enriched leather. Because an insole may remain in prolonged contact with the foot, cytocompatibility and dermal safety are essential considerations. Future studies should therefore include cytotoxicity testing using appropriate skin-cell models and, where justified, dermal irritation and sensitization assessments.

Third, the present work did not determine the release kinetics of the phytochemicals from the leather matrix. Time-dependent extraction and diffusion studies are required to establish whether active constituents are rapidly released, gradually released, or strongly retained within the leather. Such information would be important for determining the expected duration of antimicrobial activity.

Fourth, the durability of the antimicrobial function was not assessed. Future investigations should examine antimicrobial performance following repeated flexing, abrasion, exposure to artificial sweat, moisture, washing or cleaning cycles, and simulated footwear use. Measurements before and after these treatments would provide evidence regarding the stability of the formulation.

Fifth, although the GC–MS results provide useful chemical characterization, several compounds were identified tentatively. Confirmation using authentic standards, retention indices, mass-spectral match quality,



and procedural blanks would improve confidence in compound identification. Particular attention should be given to the reported bis(2-ethylhexyl) phthalate in *C. officinalis* and the ambiguous compound assignments in *B. pinnatum*.

Finally, the study did not directly assess the mechanical or pressure-relieving performance of the leather insoles. Because off-loading is an important component of diabetic foot management, future work should combine antimicrobial testing with plantar-pressure mapping, mechanical compression testing, material durability assessment, and appropriate biomechanical evaluation. Subsequent *in vivo* studies could then examine safety and biological performance before carefully designed clinical investigations are considered. Overall, the findings establish a preliminary scientific basis for developing phytochemical-enriched leather as an antimicrobial footwear material. Further multidisciplinary investigation integrating microbiology, analytical chemistry, materials science, biomechanics, toxicology, and clinical wound-care research is required to determine whether the material can provide reproducible and safe performance under realistic use conditions.

5.0 Conclusion

This study demonstrated the feasibility of developing phytochemical-enriched leather insoles with measurable *in vitro* antimicrobial activity using ethanolic extracts of *Bryophyllum pinnatum*, *Calendula officinalis*, and *Althaea officinalis*. Phytochemical screening revealed the presence of alkaloids, cardiac glycosides, saponins, phenolic compounds, tannins, steroids, carbohydrates, flavonoids, and terpenoids in the extracts, while anthraquinones were not detected. The crude extracts exhibited antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Candida albicans*, with *C. officinalis* showing the highest inhibition

against *S. aureus* (42 mm; MIC = 0.10 mg/mL; MBC = 0.20 mg/mL).

Following incorporation into leather, the extracts retained measurable antimicrobial activity, although inhibition zones were generally lower than those obtained with the corresponding crude extracts. The enriched leather therefore demonstrated potential as a carrier material for plant-derived antimicrobial constituents. GC–MS analysis further revealed diverse lipid-related constituents, particularly fatty acid esters, while FTIR analysis showed functional-group profiles consistent with the presence of organic constituents in the extracts and chemical differences between untreated leather and extract-containing formulations.

The findings provide a preliminary basis for the development of antimicrobial leather insole materials that could potentially complement conventional diabetic foot-care strategies. However, the present study was limited to laboratory-based antimicrobial and physicochemical evaluation and did not establish clinical efficacy, wound-healing activity, plantar-pressure reduction, long-term antimicrobial durability, or skin safety. Further studies should therefore investigate phytochemical release kinetics, cytocompatibility, dermal safety, mechanical durability, antimicrobial stability under simulated footwear conditions, pressure-distribution characteristics, and *in vivo* and clinical performance. Thus, the developed phytochemical-enriched leather insoles should presently be regarded as a promising research-stage antimicrobial footwear material, rather than a clinically validated treatment for diabetic foot ulcers.

6.0 References

- Afanasyeva, P. V., Kurkina, A. V., Kurkin, V. A., Lyamin, A. V., & Zhestkov, A. V. (2016). Determination of antimicrobial activity of extracts of *Calendula officinalis* flowers. *Pharmacy & Pharmacology*, 4(2),



- 60–70. [https://doi.org/10.19163/2307-9266-2016-4-2\(15\)-60-70](https://doi.org/10.19163/2307-9266-2016-4-2(15)-60-70)
- Ausloos, P., Clifton, C. L., Lias, S. G., Mikaya, A. I., Stein, S. E., Tchekhovskoi, D. V., Tchekhovskoi, D. V., & Zhu, D. (1999). The critical evaluation of a comprehensive mass spectral library. *Journal of the American Society for Mass Spectrometry*, 10(4), 287–299.
- Camilleri Attard, F., Gatt, A., & Formosa, C. (2024). Superficial tissue swabs versus deep tissue samples in the detection of microbiological profile of infected diabetic foot ulcerations. *International Journal of Lower Extremity Wounds*, 23(2), 315–319.
- Clinical and Laboratory Standards Institute. (2020). *Performance standards for antimicrobial susceptibility testing* (30th ed., CLSI supplement M100). Clinical and Laboratory Standards Institute.
- Clinical and Laboratory Standards Institute. (2024). *Performance standards for antimicrobial disk susceptibility tests* (14th ed., CLSI standard M02). Clinical and Laboratory Standards Institute.
- Dhingra, G., Dhakad, P., & Tanwar, S. (2022). Review on phytochemical constituents and pharmacological activities of plant *Calendula officinalis* Linn. *Biological Sciences*. <https://doi.org/10.55006/biolsciences.2022.2205>
- Dimri, A. G., Chaudhary, S., Singh, D., Chauhan, A., & Aggarwal, M. (2020). Morphological and biochemical characterization of food borne gram-positive and gram-negative bacteria. *Scientific Archives*, 1(1), 16–23.
- Do, N. H., Huynh, T. N., Le, T. X., Ha, A. C., & Le, P. K. (2023). Encapsulation of *Triphasia trifolia* extracts by pH and thermal dual-sensitive chitosan hydrogels for controlled release. *Carbohydrate Polymers*, 320, 121264.
- Hamad, M. N., Mohammed, H. J., & Merdaw, M. A. (2017). Antibacterial activity of *Calendula officinalis* flowers in vitro. *Ibn Al-Haitham Journal for Pure and Applied Science*, 24(3). <https://doi.org/10.30526/24.3.735>
- He, X., Li, Y., Chen, Y., Lu, J., Zhang, W., Ning, M., & Han, Z. (2026). The efficacy of three antimicrobial dressings in the management of diabetic foot ulcers: A systematic review and network meta-analysis. *Frontiers in Endocrinology*, 17, 1780580. <https://doi.org/10.3389/fendo.2026.1780580>
- Hossain, M. M., Ahmed, S., Zaman, S., Bari, M. L., & Roy, U. K. (2022). *Polygonum hydropiper* in goatskin preservation: A sustainable leather processing approach. *Sustainable Chemistry and Pharmacy*, 29, 100770.
- Hufsky, F., & Böcker, S. (2017). Mining molecular structure databases: Identification of small molecules based on fragmentation mass spectrometry data. *Mass Spectrometry Reviews*, 36(5), 624–633.
- Humphries, R. M., Abbott, A. N., & Hindler, J. A. (2019). Understanding and addressing CLSI breakpoint revisions: A primer for clinical laboratories. *Journal of Clinical Microbiology*, 57(6), e00203-19.
- Ibikunle, I. A., Bolanle, K. S., Jumai, A. A., Ifeoluwa, D. G., Anibijuwon, I. I., Saliu, B. K., Abioye, J. A., & Gbala, I. D. (2017). Antimicrobial activities of *Bryophyllum pinnatum* on some selected clinical isolates. *Fountain Journal of Natural and Applied Sciences*, 6(1), 1–8.
- Jyotisree, G., Sruthi, R., Biju, C. R., & Menon, A. S. (2020). *Calendula officinalis* and *Echinacea purpurea* as antimicrobial agent. *Journal of Applied Pharmaceutical Research*, 8(2), 002.



- <https://doi.org/10.18231/j.joapr.2020.v.8.i.2.002>
- Kandasamy, S., & Alagarsamy, S. (2025). Phytochemical-based therapeutics for diabetic foot ulcer management: Mechanistic insights and advances in wound healing strategies. *Journal of Pharmacognosy and Phytochemistry*, 14(6), 211–226. <https://doi.org/10.22271/phyto.2025.v14.i6c.15660>
- Kasumbwe, K., Odun-Ayo, F., & Mohanlall, V. (2026). Innovative phytochemical-based approaches for the treatment of diabetic foot ulcers. *Current Diabetes Reviews*, 22. Advance online publication. <https://doi.org/10.2174/0115733998431722260327100634>
- Krawiec, A., Czerwińska-Ledwig, O., Kita, B., & Piotrowska, A. (2023). Active ingredients and natural raw materials used in foot care in diabetic patients—A literature review. *Cosmetics*, 10(4), 96. <https://doi.org/10.3390/cosmetics10040096>
- Lazzarini, P. A., & Jarl, G. (2021). Knee-high devices are gold in closing the foot ulcer gap: A review of offloading treatments to heal diabetic foot ulcers. *Medicina*, 57(9), 941.
- Levine, N. S., Lindberg, R. B., Mason, A. D., Jr., & Pruitt, B. A., Jr. (1976). The quantitative swab culture and smear: A quick, simple method for determining the number of viable aerobic bacteria on open wounds. *Journal of Trauma*, 16(2), 89–94. <https://doi.org/10.1097/00005373-197602000-00002>
- Liu, Q., Liu, X., Fan, L., Bai, X., Pan, H., Luo, H., & Bowen, C. R. (2024). Ferroelectric catalytic BaTiO₃-based composite insoles to promote healing of infected wounds: Analysis of antibacterial efficacy and angiogenesis. *Interdisciplinary Materials*, 3(5), 757–774.
- Oladunmoye, M. K., Afolami, O. I., Oladejo, B. O., Amoo, I. A., & Osho, B. I. (2019). Identification and quantification of bioactive compounds present in the plant *Vernonia amygdalina* Delile using GC-MS technique. *Natural Products Chemistry & Research*, 7, 356.
- Ramsay, S., Cowan, L., Davidson, J. M., Nanney, L., & Schultz, G. (2016). Wound samples: Moving towards a standardised method of collection and analysis. *International Wound Journal*, 13(5), 880–891.
- Ren, Y., Wang, H., Song, X., Wu, Y., Lyu, Y., & Zeng, W. (2024). Advancements in diabetic foot insoles: A comprehensive review of design, manufacturing, and performance evaluation. *Frontiers in Bioengineering and Biotechnology*, 12, 1394758. <https://doi.org/10.3389/fbioe.2024.1394758>
- Rezaei, M., Dadgar, Z., Noori-Zadeh, A., Mesbah-Namin, S. A., Pakzad, I., & Davodian, E. (2015). Evaluation of the antibacterial activity of the *Althaea officinalis* L. leaf extract and its wound healing potency in the rat model of excision wound creation. *Avicenna Journal of Phytomedicine*, 5(2), 105–112.
- Suryawanshi, S., Kumar, A., Deshmukh, D., Jain, A., Tiwari, A., Vaishya, B. P., Singh, B. K., Ravindra, C. S., & Rathi, J. C. (2025). Phytochemical screening and antimicrobial activity of *Bryophyllum pinnatum* leaf extract. *World Journal of Biology, Pharmacy and Health Sciences*, 22(1), 482–486. <https://doi.org/10.30574/wjbpshs.2025.22.1.0301>
- Suraya, A. S., Jannah, F., Sriyono, S., & Armini, N. K. A. (2025). Use of insole design footwear to prevent risk of foot ulcers in diabetes patients: A systematic review. *Indonesian Nursing Journal of*



- Education and Clinic (INJEC)*, 9(2).
<https://doi.org/10.24990/injec.v9i2.670>
- Teixeira, I. D., Carvalho, E., & Leal, E. C. (2023). Green antimicrobials as therapeutic agents for diabetic foot ulcers. *Antibiotics*, 12(3), 467.
<https://doi.org/10.3390/antibiotics12030467>
- Wang, Q., Liu, C., An, J., Liu, J., Wang, Y., & Cai, Y. (2025). Mechanisms of microbial infection and wound healing in diabetic foot ulcer: Pathogenicity in the inflammatory-proliferative phase, chronicity, and treatment strategies. *Frontiers in Endocrinology*, 16, 1657928.
- Kasumbwe, K., Odun-Ayo, F., & Mohanlall, V. (2026). Innovative phytochemical-based approaches for the treatment of diabetic foot ulcers. *Current Diabetes Reviews*, 22. Advance online publication.
<https://doi.org/10.2174/0115733998431722260327100634>
- Suraya, A. S., Jannah, F., Sriyono, S., & Armini, N. K. A. (2025). Use of insole design footwear to prevent risk of foot ulcers in diabetes patients: A systematic review. *Indonesian Nursing Journal of Education and Clinic (Injec)*, 9(2).
<https://doi.org/10.24990/injec.v9i2.670>
- Kandasamy, S., & Alagarsamy, S. (2025). Phytochemical-based therapeutics for diabetic foot ulcer management: Mechanistic insights and advances in wound healing strategies. *Journal of Pharmacognosy and Phytochemistry*, 14(6), 211–226.
<https://doi.org/10.22271/phyto.2025.v14.i6c.15660>
- He, X., Li, Y., Chen, Y., Lu, J., Zhang, W., Ning, M., & Han, Z. (2026). The efficacy of three antimicrobial dressings in the management of diabetic foot ulcers: A systematic review and network meta-analysis. *Frontiers in Endocrinology*, 17, Article 1780580.
<https://doi.org/10.3389/fendo.2026.1780580>
- Ren, Y., Wang, H., Song, X., Wu, Y., Lyu, Y., & Zeng, W. (2024). Advancements in diabetic foot insoles: A comprehensive review of design, manufacturing, and performance evaluation. *Frontiers in Bioengineering and Biotechnology*, 12, Article 1394758.
<https://doi.org/10.3389/fbioe.2024.1394758>
- d in Foot Care in Diabetic Patients—A Literature Review. *Cosmetics*, 10(4), 96.
<https://doi.org/10.3390/cosmetics10040096>

Compliance with Ethical Standards Declaration

Ethical Approval

Not Applicable

Competing interests

The author declares no known competing financial interests.

Funding

No external funding was received for this doctrinal and comparative legal research.

Author's Contribution

Akabuogu Ebere Promise conceptualized the study, conducted experiments, analyzed data, and drafted the manuscript. Oyewale Adebayo Ojo contributed to methodology, experiments, data analysis, and manuscript revision. Habila James Dama contributed to phytochemical and antimicrobial investigations and interpretation of results. Hamisu Ibrahim supervised the research, contributed to data interpretation, critically reviewed the manuscript, and coordinated the study. All authors reviewed and approved the final manuscript.

