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ORIGINAL ARTICLE

Analytical Standardization and In-Vitro Antimicrobial Assessment of a Herbal Formulation: V Fresh Spray

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ABSTRACT
V Fresh Spray is an Ayurvedic polyherbal formulation, commonly used for its antimicrobial properties. In the current study, HPTLC (High-Performance Thin Layer Chromatography) and in-vitro antimicrobial activity testing were employed to analyze and validate its phytochemical profile and antimicrobial effectiveness against Pseudomonas aeruginosa and Candida albicans. To establish a reproducible HPTLC fingerprint for V Fresh Spray and evaluate its in-vitro antibacterial and antifungal activity. HPTLC analysis was conducted using Merck HPTLC Silica Gel 60 F254 plates with a mobile phase of Toluene: Ethyl acetate: Formic acid (5:4:1 v/v/v). Detection was performed at 254 nm (absorbance) and 366 nm (fluorescence). Antimicrobial activity was assessed using the Cylinder Plate Method as per the Indian Pharmacopoeia 2014, against P. aeruginosa ATCC 9027 and C. albicans ATCC 10231. The HPTLC analysis revealed multiple distinct phytochemical zones, with a dominant peak at Rf ~0.72–0.75, suggesting a major phytochemical constituent. Fluorescence detection showed additional peaks indicative of compounds such as flavonoids and phenolics. In-vitro testing demonstrated a 6 mm zone of inhibition against P. aeruginosa and a 7 mm zone against C. albicans, indicating moderate antibacterial and good antifungal activity. The study establishes a reproducible HPTLC fingerprint of V Fresh Spray and demonstrates measurable antimicrobial activity. These findings provide preliminary validation for the formulation and support further pharmacological and quantitative research to substantiate its therapeutic efficacy.
Keywords: *HPTLC fingerprinting; Herbal formulation; V Fresh Spray; Antimicrobial activity; Phytochemical profiling; Cylinder Plate Method*

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INTRODUCTION

Herbal formulations have been widely used in traditional systems of medicine for the prevention and management of microbial infections. Classical references in Ayurveda describe various herbs with antimicrobial properties, such as Karanja (*Pongamia pinnata*) [1], Shigru Patra (*Moringa oleifera*) [2], and Laksha (*Laccifer lacca*) [3], which have been used for centuries to treat infections. With the growing concern of antimicrobial resistance and side effects associated with synthetic antibiotics, there is increasing interest in scientifically validating polyherbal preparations. Analytical standardization plays a crucial role in ensuring batch-to-batch consistency, safety, and therapeutic reliability of herbal products. V Fresh Spray is a polyherbal formulation intended for topical antimicrobial applications. The composition includes herbs known for their antimicrobial, anti-inflammatory, and antioxidant properties, and its use is supported by ancient Ayurvedic texts, although scientific validation is necessary for confirming its efficacy in modern clinical contexts. Systematic analytical fingerprinting, such as High-Performance Thin Layer Chromatography (HPTLC), and scientific validation of its antimicrobial activity are essential for establishing its quality and efficacy. HPTLC is a well-accepted analytical technique for

profiling the phytochemical constituents of complex herbal formulations due to its sensitivity, reproducibility, and cost-effectiveness.
The present study was designed to generate an HPTLC fingerprint profile of V Fresh Spray and evaluate its in-vitro antimicrobial activity against representative bacterial and fungal strains.

MATERIAL AND METHODS

Formulation Details

Sample Used: The formulation used in this study was **V Fresh Spray**. The analysis was conducted at a **Ministry of AYUSH Approved Ayurvedic Testing Laboratory**.
Batch Number: Nov. 2025
Manufacturer: Parul Institute of Ayurved and Research Pharmacy
Preparation of Extracts:
Shigru Patra (*Moringa oleifera*):
Alcoholic Extract: 1 part Shigru Patra with 10 parts ethyl alcohol
Karanja (*Pongamia pinnata*):
Hydroalcoholic Extract: 1 part Karanja with 4 parts methyl alcohol + 6 parts distilled water
Laksha (*Laccifer lacca*):
Water Extract: 1 part Laksha with 10 parts ethyl alcohol
Samples for Testing:
First Sample: Equal proportion of Shigru Patra, Karanja, and Laksha
Second Sample: Half proportion of Shigru Patra, Karanja, and Laksha
Third Sample: Double proportion of Shigru Patra, Karanja, and Laksha
Methodology for Preparation of the Spray:
The proportion of each drug was evaluated to identify the combination with the best Minimum Inhibitory Concentration (MIC) value. The combination with the highest MIC was chosen for the preparation of the spray.

- After selecting the drug combination with the highest MIC, the extract was processed with eophilisers (CDM 10%).
- The mixture was homogenized for 24 hours and placed in a dark chamber for one day to ensure proper blending of the active constituents.
- The prepared mixture was then combined with:

0.5% Methyl Paraben
8 drops of Tea Tree Oil
1% Hydrogenated Glycerine

- The mixture was stirred with a magnetic stirrer for 30 minutes to ensure uniform dispersion of the active ingredients.
- After homogenization and testing, the mixture was assessed for sprayability and drug dispersion.
- Once satisfactory results were achieved, the final formulation was filled into sterile spray bottles for further testing.

Microbial Strains:
The following standard strains were used for antimicrobial testing:

- *Pseudomonas aeruginosa* ATCC 9027
- *Candida albicans* ATCC 10231

Media Used

- Mueller Hinton Agar was used for antibacterial testing.
- Sabouraud Dextrose Agar was used for antifungal testing.

Method for Antimicrobial Activity
The antimicrobial activity was determined using the Cylinder Plate Method as per Indian Pharmacopoeia 2014, Chapter 2.2.10. Freshly prepared slants of the respective microorganisms were washed with sterile normal saline to prepare inoculum suspensions. Sterile media were cooled to approximately 55°C and inoculated with 10 µL of the respective microbial cultures. The inoculated media were poured into sterile Petri plates and allowed to solidify. Wells were made using a sterile borer, and the test sample was directly added into the labeled wells. The plates were incubated at 35°C for 24 hours for bacterial culture and at 25°C for 48 hours for fungal culture. Zones of inhibition were measured in millimeters.
HPTLC Analysis
HPTLC analysis was performed on Merck HPTLC Silica Gel 60 F254 plates (100 × 100 mm). The mobile phase consisted of Toluene: Ethyl acetate: Formic acid in the ratio 5:4:1 v/v/v. The chamber was

saturated for 20 minutes before development. Sample application was performed using Linomat 5, and the plates were developed up to a solvent front of 80 mm.
Densitometric scanning was carried out using TLC Scanner 4 at 254 nm in absorbance mode and at 366 nm in fluorescence mode. Four application volumes were analyzed: 5 µL, 10 µL, 15 µL, and 20 µL.

RESULT

HPTLC Fingerprinting at 254 nm

Multiple distinct phytochemical zones were observed across application volumes. A dominant peak (~0.92 Rf) was consistently observed, contributing approximately 57–62% of the total peak area.

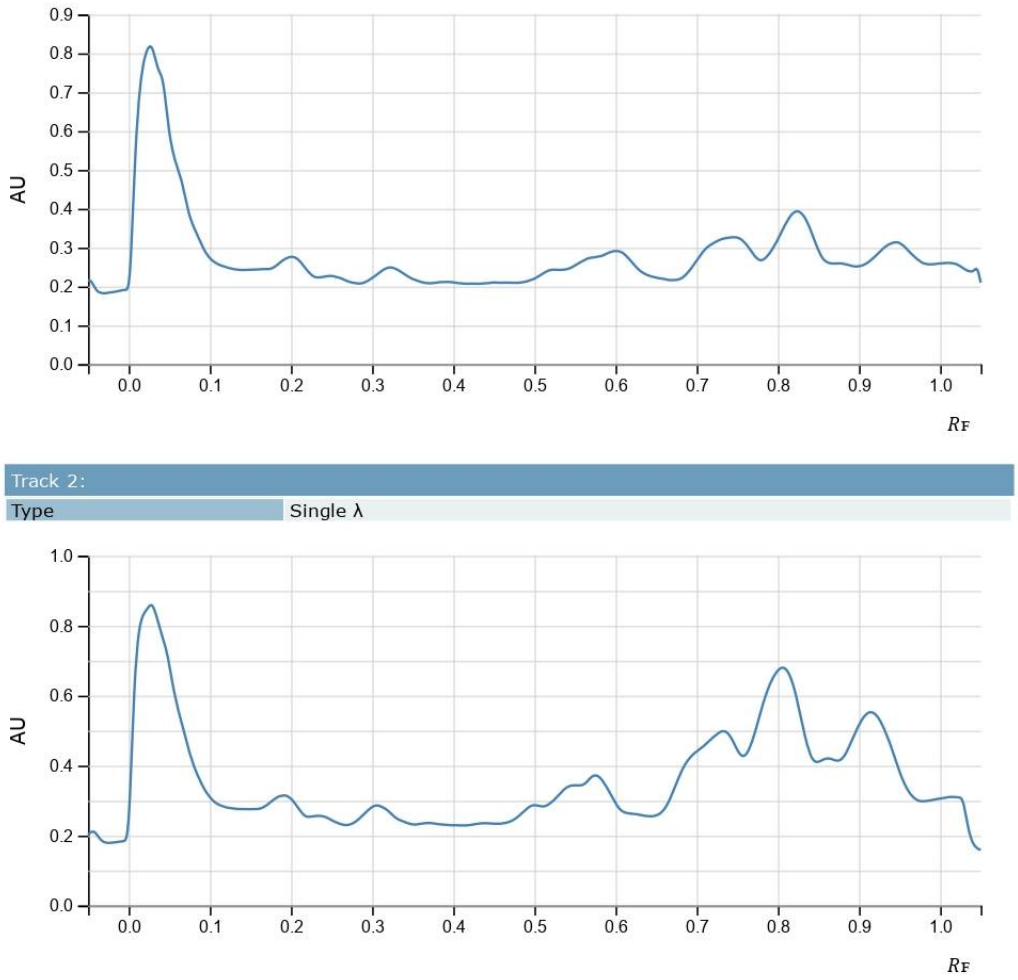

Figure 1. Densitometric chromatograms of V Fresh Spray at 254 nm showing Track 1 (5 µL) and Track 2 (10 µL).

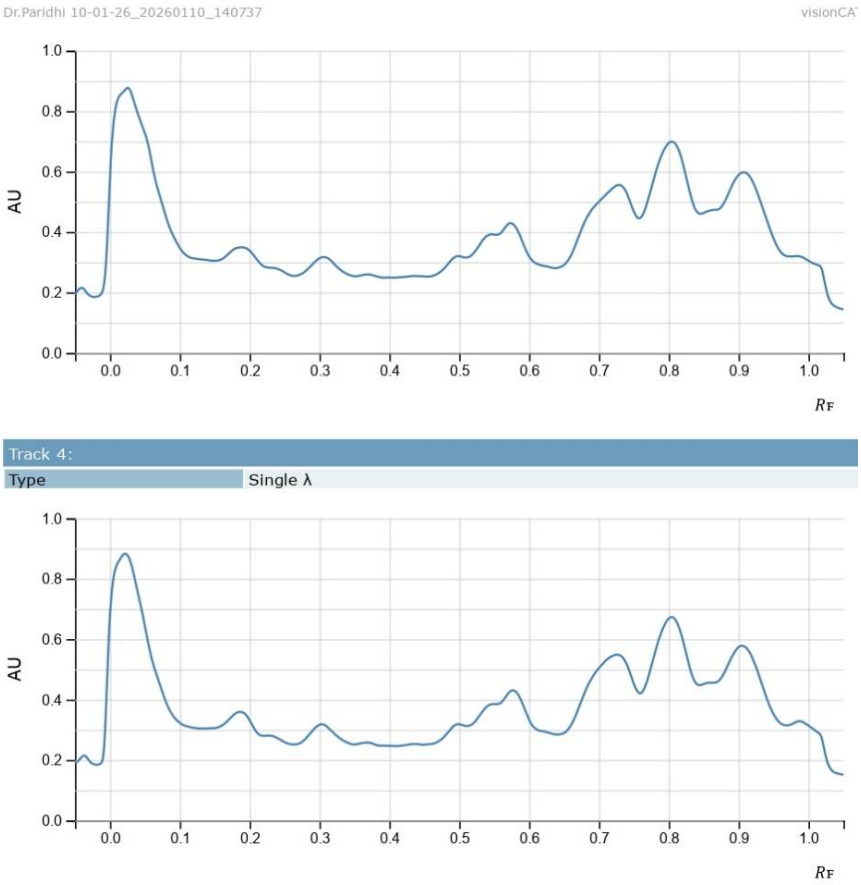

Figure 2. Densitometric chromatograms of V Fresh Spray at 254 nm showing Track 3 (15 µL) and Track 4 (20 µL).

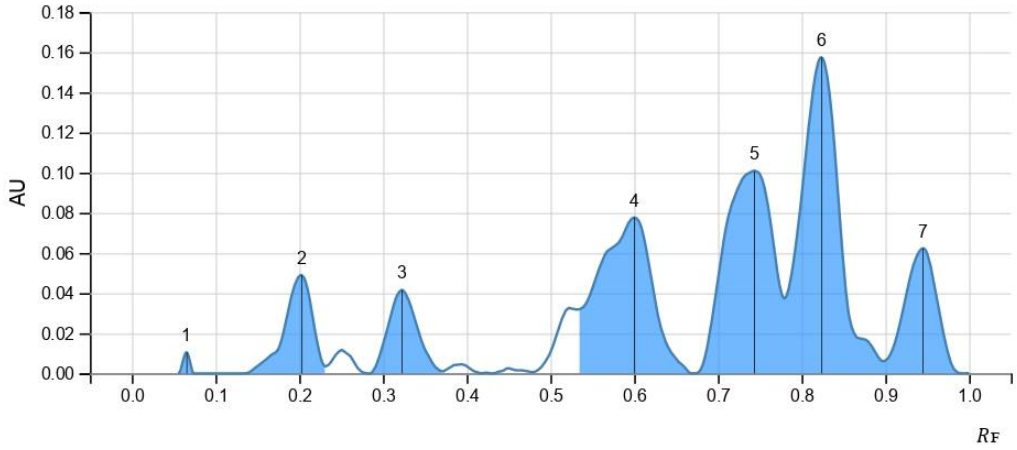

Figure 3. Peak profile at 254 nm (5 µL) showing Rf values and peak area distribution.

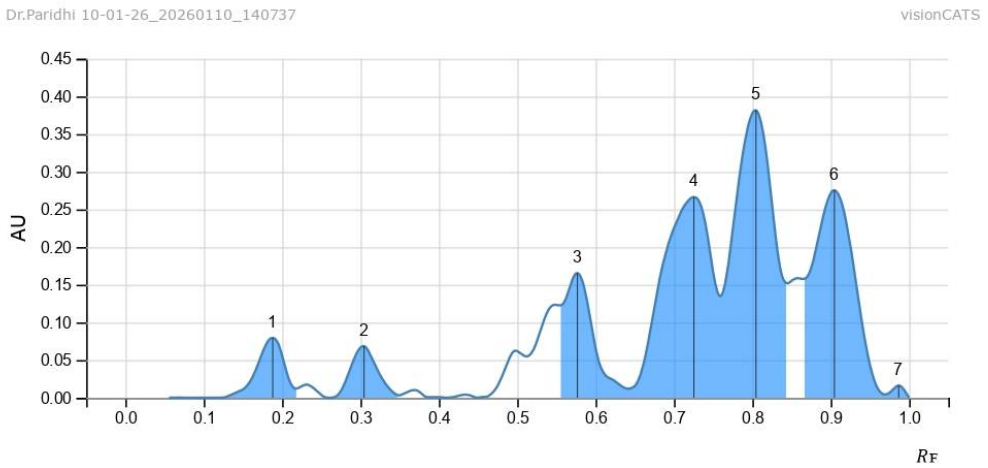

Figure 4. Peak profile at 254 nm (20 µL) showing major and minor peaks with Rf values and area percentages.

HPTLC Fingerprinting at 366 nm

At 366 nm, fluorescence detection revealed additional bands, with a dominant band at ~0.75–0.80 Rf contributing to a significant portion of the sample's profile.

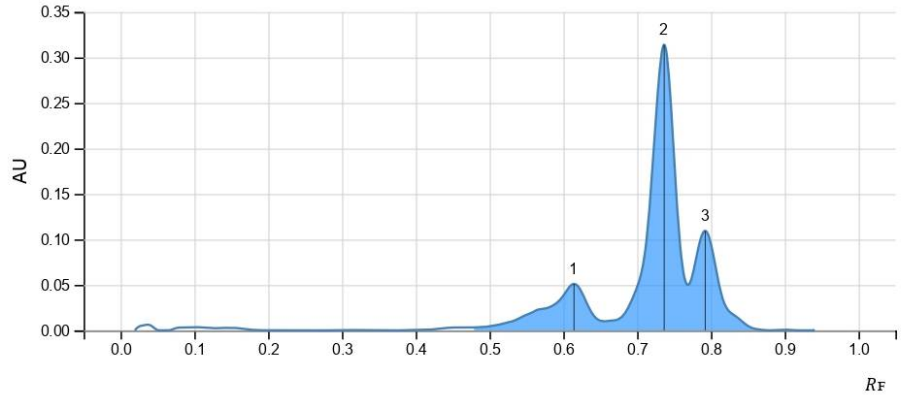

Figure 5. Densitometric chromatogram of V Fresh Spray at 366 nm (5 µL) showing fluorescent peak distribution.

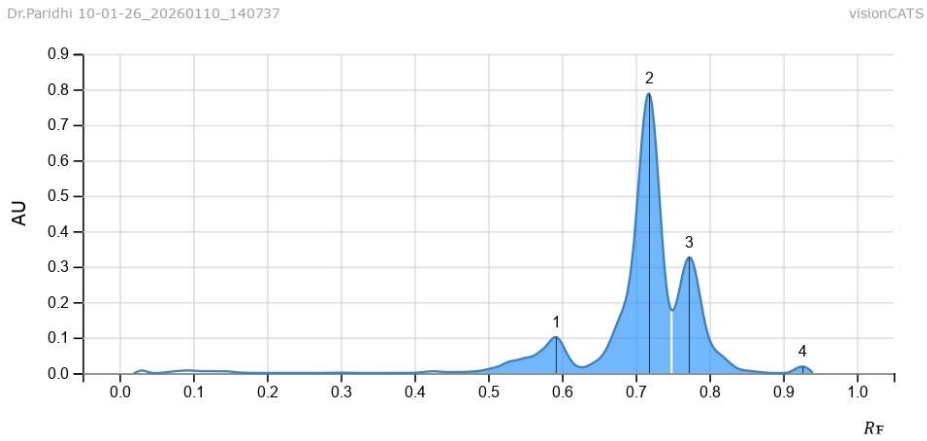

Figure 6. Densitometric chromatogram of V Fresh Spray at 366 nm (20 µL) showing fluorescent peak distribution.

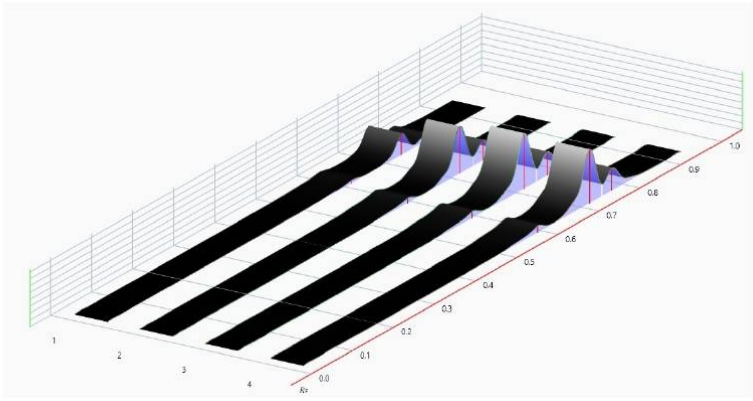

Figure 7. Three-dimensional densitometric profile of V Fresh Spray at 366 nm illustrating fluorescent peak distribution.

In-Vitro Antibacterial and Antifungal Activity

The results of the antimicrobial assay showed measurable inhibition zones.
Against *Pseudomonas aeruginosa*, the formulation produced a zone of inhibition measuring 6 mm. Against *Candida albicans*, the zone of inhibition measured 7 mm.
These findings indicate moderate antibacterial activity and comparatively better antifungal activity under the tested conditions.

Table 1. *In-vitro* antimicrobial activity of V Fresh Spray by Cylinder Plate Method (Indian Pharmacopoeia 2014)

Sample	*Pseudomonas aeruginosa* ATCC 9027	*Candida albicans* ATCC 10231
V Fresh Spray	6 mm	7 mm

DISCUSSION

The present study aimed to establish an analytical fingerprint profile and evaluate the antimicrobial potential of the polyherbal formulation V Fresh Spray. Standardization of herbal formulations is a crucial requirement in modern phytopharmaceutical research to ensure batch-to-batch consistency, safety, and therapeutic reliability. Chromatographic fingerprinting using High Performance Thin Layer Chromatography (HPTLC) has emerged as a widely accepted analytical tool for qualitative and semi-quantitative assessment of phytochemical constituents in complex herbal formulations due to its high sensitivity, reproducibility, and ability to analyze multiple samples simultaneously [4,5]. The HPTLC chromatogram obtained in the present investigation demonstrated multiple distinct peaks distributed across a wide Rf range, indicating the presence of diverse phytochemical constituents within the formulation. Such multicomponent chromatographic patterns are typical of polyherbal preparations containing several plant-derived metabolites. Similar chromatographic diversity has been reported in previous studies on herbal formulations where HPTLC fingerprinting was used for phytochemical profiling and quality control [6]. The presence of multiple peaks suggests that the formulation contains a complex mixture of secondary metabolites including phenolics, flavonoids, terpenoids, and other bioactive constituents. Among the detected peaks, a dominant peak was observed in the mid-Rf region (approximately 0.72–0.75 at 254 nm), which accounted for a significant proportion of the total peak area. The reproducibility of this peak across different sample volumes indicates that it represents a major phytochemical component of the formulation. Such prominent peaks are often considered potential analytical markers for quality control and standardization of herbal formulations [7]. Identification and characterization of this compound through advanced analytical techniques such as HPLC-MS or LC-MS may provide further insights into the bioactive principles responsible for the therapeutic effects of the formulation. Fluorescence detection at 366 nm revealed additional peaks that were either absent or less prominent at 254 nm. This observation highlights the advantage of dual-wavelength scanning in HPTLC analysis, which enhances the detection of certain classes of compounds that exhibit fluorescence under ultraviolet light. The intense fluorescent peaks observed in the mid-Rf region may correspond to phytochemical groups such as flavonoids, coumarins, or other phenolic compounds. These classes of

secondary metabolites are widely reported in medicinal plants and have been associated with significant pharmacological activities including antimicrobial, antioxidant, and anti-inflammatory effects [8]. The antimicrobial assay conducted in this study demonstrated measurable inhibitory activity of the formulation against both bacterial and fungal test organisms. The zone of inhibition of 6 mm observed against *Pseudomonas aeruginosa* suggests the presence of bioactive constituents capable of exerting antibacterial effects. *Pseudomonas aeruginosa* is a Gram-negative bacterium known for its intrinsic resistance to many antimicrobial agents due to the presence of an outer membrane barrier and various efflux mechanisms [9]. Therefore, the ability of the formulation to produce measurable inhibition against this organism indicates the potential presence of compounds capable of interfering with bacterial growth or cellular metabolism. The formulation exhibited a slightly higher inhibition zone of 7 mm against *Candida albicans*, suggesting comparatively better antifungal activity. Fungal pathogens are often susceptible to phytochemicals such as phenolic compounds, flavonoids, and terpenoids that disrupt fungal cell membrane integrity, inhibit enzymatic pathways, or interfere with oxidative processes [10]. The stronger antifungal response observed in the present study may therefore be associated with the presence of such phytochemical constituents detected in the chromatographic analysis. The antimicrobial potential observed in the formulation may also be attributed to the pharmacologically active constituents present in its individual herbal ingredients. Previous studies have demonstrated that *Moringa oleifera* possesses significant antimicrobial and antioxidant properties due to the presence of flavonoids, phenolic acids, and other bioactive compounds [11]. Similarly, *Pongamia pinnata* has been reported to contain bioactive flavonoids and furanoflavonoids such as karanjin that exhibit antibacterial and antifungal activities [12]. The combined presence of such phytoconstituents in a polyherbal formulation may contribute to synergistic antimicrobial effects. Despite the promising findings, the present investigation has certain limitations that should be acknowledged. The antimicrobial evaluation was limited to the measurement of zones of inhibition using the cylinder plate method. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) would provide a more precise assessment of antimicrobial potency. Furthermore, the study evaluated activity against only one bacterial and one fungal strain. Testing against a broader panel of clinically relevant microorganisms would strengthen the pharmacological relevance of the findings. Nevertheless, the present study successfully establishes a reproducible chromatographic fingerprint profile and provides preliminary evidence of antimicrobial activity for the polyherbal formulation. The combination of analytical standardization and biological evaluation represents an important step toward scientific validation of traditional herbal preparations and supports their potential application in antimicrobial therapy.

LIMITATIONS

The study did not include identification of specific marker compounds responsible for antimicrobial activity. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration were not determined. Only single bacterial and fungal strains were tested. No positive antibiotic control was included for comparative analysis. Statistical validation and replicate analysis were not performed.

CONCLUSION

The present study establishes a reproducible HPTLC fingerprint profile of V Fresh Spray and demonstrates measurable *In-vitro* antimicrobial activity against Pseudomonas aeruginosa and *Candida albicans*. The dominant mid-Rf chromatographic peak may serve as a potential analytical marker for quality control and future standardization. Although the antimicrobial activity observed was moderate, the findings provide preliminary scientific validation of the formulation. Further pharmacological, phytochemical, and quantitative studies are recommended to substantiate its therapeutic potential and enhance its standardization framework.

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