

LC–MS-Based Characterization and Antioxidant Profiling of an Ethanolic Extract of *Calotropis procera*

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Abstract

Objective: The present investigation aimed to establish the phytochemical profile of *Calotropis procera* (Aiton) W.T. Aiton extract by applying complementary chromatographic and mass-spectrometric techniques and to evaluate its phenolic content as a preliminary estimation of its possible application to antioxidant-related activity. An LC–MS metabolite profile was generated to aid the identification of candidate bioactive compounds for further study, with particular emphasis given to the identification and characterization of metabolites. **Methods:** Phytochemical screening and determination of total phenolic content (TPC), high-performance thin-layer chromatography (HPTLC) and liquid chromatography-mass spectrometry (LC-MS) analysis were performed on the extract. The TPC obtained was 14.68 ± 0.42 mg of Gallic acid equivalents/g of dry extract. The chromatograms obtained by HPTLC showed a complex pattern with several distinct bands, with a prominent band at R_f 0.43, which matched the R_f value of the chelidone reference standard under the conditions used for the HPTLC analysis. The detected profile was chemically diverse, with significant features being present at m/z 573.17, 570.08 and 515.17, respectively, having retention times of ~ 18.38 , ~ 20.95 and ~ 18.95 min. The results suggest the extract contained several different chemically distinct secondary metabolites. **Discussion:** The results obtained from the combined use of the HPTLC and LC–MS indicate that *C. procera* extract is a complex chemical mixture of secondary metabolites along with the presence of measurable amounts of phenols. The observed phenolic content offers an initial basis to explore its potential for antioxidant activity; in this regard, the results obtained from the present analyses cannot be used to prove antioxidant activity or the antioxidant contribution of individual metabolites. The LC–MS profile is helpful in guiding metabolite isolation, confirmation, quantitative characterization, and bioactivity studies. Further investigation is warranted to determine the correlation between individual constituents and the experimentally determined antioxidant and pharmacological activities.

Keywords: *Calotropis procera*; LC–MS; phytochemical profiling; antioxidant activity; total phenolic content; HPTLC; chelidone; secondary metabolites.

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INTRODUCTION

Medicinal plant species in many parts of the world. They are rich in non-nutritive and nutritive compounds, including essential oils, vitamins and minerals, and contain high concentrations of bioactive compounds like flavonoids [1] Phenolics, anthocyanins and phenolic acids. The study has shown that the crude plant species have immense medicinal and health care benefits, as their use in ancient times for medicinal, religious, cosmetic, nutritional, and beautification purposes is well documented [2]. Under physiological conditions, reactive oxygen species (ROS) like superoxide anion, hydroxyl radicals and hydrogen peroxide are constantly generated, and they are additionally formed as a result of environmental exposure. Oxidative stress from these reactive species can lead to a disruption of redox homeostasis, causing lipid,

protein and nucleic acid oxidative damage, which can play a role in the onset and development of various chronic diseases[3]. Thus, it has become of paramount concern in the field of pharmaceutical and natural-product research to identify naturally derived antioxidants that can remove the reactive species or regulate the oxidative pathways. Plant extracts are especially appealing because the antioxidant activity they possess is frequently linked to a variety of phenolic acids, flavonoids, terpenoids and other secondary metabolites that could work together in a complementary way[4]. *Calotropis Procera* (Aiton) W.T. Aiton, also referred to as *Calotropis* or giant milkweed, is a medicinal plant that belongs to the Apocynaceae family and is widely distributed in tropical and subtropical zones. Various organs of [5] *C. Procera*, such as leaves,

roots, bark, flowers, and latex, have been traditionally studied for their different pharmacological activities. The phytochemical studies have revealed the presence of various classes of secondary metabolites, including flavonoids, phenolics, terpenoids, glycosides and other bioactive compounds[6]. This chemical diversity offers a rational approach to exploring the possibility of finding molecules with pharmacologic relevance in *C. Procera*. Different experimental models have been used to demonstrate the antioxidant properties of *C. Procera*. Ethanol extract of the plant has been found to possess free-radical-scavenging properties, and *C. Procera* extracts are associated with significant amounts of phenolics and flavonoids with antioxidant responses[7]. In particular, ethanolic extracts of *C. Procera* have been shown to possess DPPH radical-scavenging activity, which supports the involvement of ethanol-extractable secondary metabolites in the antioxidant activity observed. Other more recent studies have also shown correlations between phenolic/flavonoid content and antioxidant activity of *C. Procera* extracts, making the study of chemical constituents responsible for these biological activities of great importance[8].

Phytochemical screening and spectrophotometric estimation of the total phenolics and flavonoids content only give a general idea about the composition of the extract, and cannot discriminate the individual metabolites contributing to the biological activity[9]. The antioxidant potential of botanical extracts depends on the total phenolic or flavonoid content and, more specifically, on the type, amount, structural features and potential synergy of the constituents. It is therefore a necessity to understand the relationship between phytochemical composition and antioxidant activity in a meaningful way, and this requires comprehensive chemical profiling. Liquid chromatography – mass spectrometry (LC-MS) – and high-resolution LC-MS/MS, in particular, have become a very useful analytical platform for the rapid characterization of complex plant extracts[10]. Unique to this approach is the ability to identify and tentatively characterize structurally diverse metabolites in a single analytical workflow by separating them chromatographically and using accurate mass measurement and fragmentation-based structure elucidation. Several flavonoids, hydroxybenzoic acids, and hydroxycinnamic acids have been detected in *Calotropis* species using LC-MS/MS profiling, thus making mass spectrometric techniques a potent tool to elucidate the chemical complexity of this genus[11]. The same, UHPLC-QTOF-MS/MS profiling of extracts of *C. Procera* leaf has correlated the differences observed in phytochemical profile based on the type of solvent used with their antioxidant and other biological activities. Advanced mass spectrometry is still

capable of advanced investigations of phytochemical composition, including the identification of flavonoids, phenolic acids and other specialised metabolites in *C. Procera* in recent HPLC-ESI-MS/MS investigations. The chemical composition of *C. Procera* may vary significantly depending on the plant part under study, geographical region, developmental stage, extraction solvent and extraction conditions[12][13].

Discussed in this study is the limited chemical characterization of bioactive constituents responsible for the antioxidant activity of *Calotropis procera* ethanolic extract. Despite its significant ethnopharmacological value, conventional phytochemical screening provides less information about individual metabolites[14,15] and their contribution to antioxidant activity. Thus, LC-MS-based metabolite profiling is necessary to characterise the extract in a comprehensive manner and tentatively annotate its bioactive constituents. Thus, based on the above studies, the present study highlights the LC-MS chemical profile of *C. procera* ethanolic extract and its antioxidant activity assessment to find out the possible metabolites which are associated with the antioxidant activity and further scientific rationalization for the application of the extract in prescription and drug dosage forms[16].

This investigation was designed to characterize the antioxidant ethanolic extract of *Calotropis procera* by LC-MS, to identify and tentatively annotate its main metabolites according to the chromatographic and mass-spectrometric properties and to investigate the possible contribution of the metabolites identified in the extract's antioxidant properties[17].

MATERIALS AND METHODS

1.1 Plant material

Leaves of *Calotropis Procera* W.T. Aiton were plucked fresh from their plants in [Moradabad, India]. A voucher specimen was deposited and authenticated by a competent taxonomist/botanist. Carefully collected leaves were examined and were free from any soil particles or extraneous matter. The material was cleaned using distilled water, shade-dried at room temperature and kept away from direct sunlight to prevent degradation of thermolabile and photosensitive phytoconstituents[18].

1.1.1 Collection and Preparation of *Calotropis procera* Latex Hydroalcoholic Extract

The latex of *Calotropis procera* was tapped from healthy plants with a sterile scalpel by cutting into the stem in a way that does not harm the plant. The collected latex was stored in sterile amber glass containers and brought to the laboratory. Latex was thoroughly blended with 20% hydroalcoholic solvent [19] (ethanol: water, 20:80, v/v) to prepare a homogeneous mixture. After 15 min of centrifuging at 4000 rpm at room temperature, the mixture was

centrifuged to remove insoluble and coagulated materials. The supernatant was collected, filtered and kept in a dark amber bottle at 4 °C for further phytochemical, antioxidant and LC-MS analysis[20].

1.2 FTIR Spectroscopic Analysis

The compatibility of efinaconazole with the chosen formulation excipients and any possible chemical interactions during microemulsion formation were assessed using Fourier Transform Infrared (FTIR) spectroscopy. An FTIR spectrophotometer was used to record spectra of the optimised microemulsion formulation, individual excipients, and the pure medication over the spectral range 4000–400 cm^{-1} at room temperature. The potassium bromide (KBr) pellet method was used to create the samples, and the functional groups' distinctive absorption peaks were examined and contrasted[21]. The preservation of efinaconazole distinctive peaks in the optimized formulation without the emergence of additional peaks or notable peak shifts was thought to be a sign of the formulation's durability and chemical compatibility.

1.3 Differential Scanning Calorimetry (DSC)

The medication, excipients, and improved microemulsion formulation's thermal behaviour and compatibility were examined using Differential Scanning Calorimetry (DSC) analysis. Standard aluminium pans were used to precisely weigh and seal 3–5 mg of each sample, with an empty sealed pan acting as a reference. Calibrated DSC equipment was used to scan the samples over a temperature range of 25–300°C at a heating rate of 10°C/min in a nitrogen environment (50 mL/min). In order to detect distinctive endothermic and exothermic transitions, including the melting point and any modifications in thermal behaviour, thermograms were captured. Good compatibility between the drug and formulation components was thought to be indicated by the absence of notable peak changes or the emergence of new peaks in the optimized formulation[22].

1.4 Ex Vivo Transungual Permeation Study Using Porcine Hoof Membrane

Fresh porcine hooves were obtained from a licensed local slaughterhouse/source following institutional biosafety and ethical requirements. Efinaconazole, *Calotropis procera* latex, olive oil, Tween 80, glycerine, PEG 400, and distilled water were used for preparation of the optimized formulation. Phosphate-buffered saline (PBS; pH 7.4) was used as the receptor medium. Franz diffusion cells, magnetic stirrer, thermostatically controlled water bath, analytical balance, micropipettes, syringes, forceps, scalpel, surgical blade, filter paper, and suitable analytical equipment for efinaconazole estimation were used[23].

Fresh bovine (or porcine) hooves were collected from a local slaughterhouse immediately after slaughter.

Material	Specification
Fresh bovine/porcine hoof	Local slaughterhouse
Franz Diffusion Cell	Diffusion area 1.77 cm^2
Phosphate Buffer pH 7.4	Receptor medium
Methanol	Grade
Magnetic Stirrer	600 rpm
Water Bath	32 $\pm$ 0.5°C
UV Spectrophotometer	Drug estimation
Optimized Microemulsion	Test formulation
Plain Drug Solution	Control

The hooves were washed with running tap water and then distilled water to remove the dirt and tissue debris that adhered to the hooves[24,25]. A band saw was used to separate the dorsal hoof plate, and the square piece was cut (about 1.5 $\times$ 1.5 cm). The membranes were carefully thinned to a thickness of 0.40–0.60 mm using 600-grit sandpaper.

RESULTS AND DISCUSSION:

1.5 FTIR Spectra

1.5.1 FTIR spectra of formulations F1, F2, and F3

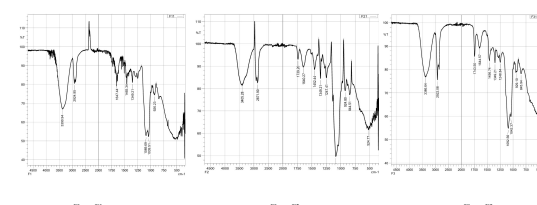


Figure 1 FTIR spectra of formulations F1, F2 and F3

The FTIR spectra of formulations F1, F2, and F3 exhibited characteristic absorption bands corresponding to O–H stretching (3386–3405 cm^{-1}), aliphatic C–H stretching (2872–2925 cm^{-1}), C=O/C=C stretching (1640–1744 cm^{-1}), CH_2 /C–H bending (1349–1466 cm^{-1}), and C–O stretching (1040–1249 cm^{-1}), along with distinct fingerprint

region peaks between 524 and 929 cm^{-1} . The preservation of these characteristic functional group peaks without significant shifts, disappearance, or the appearance of new bands indicates the absence of chemical interactions between efinaconazole and the formulation excipients. These findings confirm the excellent physicochemical compatibility, chemical stability, and integrity of the drug, demonstrating the successful development of stable microemulsion formulations (F1–F3).

1.5.2 FTIR spectra of Olive oil, Tween80, and PEG 400 (polyethylene Glycol 400):

The FTIR spectra of the formulation excipients demonstrated the characteristic functional groups of PEG (F4), Tween 80 (F5), and olive oil (F6).

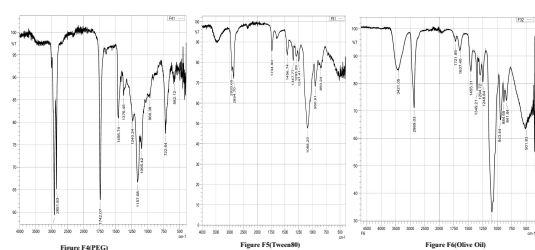


Figure 2 FTIR spectra of Polymer

The absorption bands characteristic of the polyether backbone of polyethylene glycol, around 2881 cm^{-1} , assigned to C–H stretching vibrations of the aliphatic part of the molecule, and between 1157 and 1060 cm^{-1} , as C–O–C stretching vibrations of the polyether chain, were found in the spectrum of PEG. The spectrum of Tween 80 showed a broad band at 3473 cm^{-1} corresponding to O–H stretching, a strong absorption at 1737 cm^{-1} corresponding to ester carbonyl (C=O) stretching, and strong bands between 1248 and 1062 cm^{-1} corresponding to the C–O–C stretching of polyoxyethylene sorbitan esters. The absorption bands of esters carbonyl, aliphatic C–H stretching, and C–O stretching vibrations in the region of 1238–1094 cm^{-1} were observed in olive oil, which indicates the presence of triglycerides and long-chain fatty acids. The absorption bands observed were in good agreement with the reported FTIR of these excipients. There were no peak shifts or loss of the excipients' characteristic functional groups, suggesting good chemical integrity of the excipients and no significant chemical interactions or incompatibilities between formulation components. The results showed that PEG, Tween 80, and olive oil are suitable for the development of a stable microemulsion formulation.

1.6 DSC (Differential Scanning Calorimetry): Differential Scanning Calorimetry for *Calatropis Procera* latex

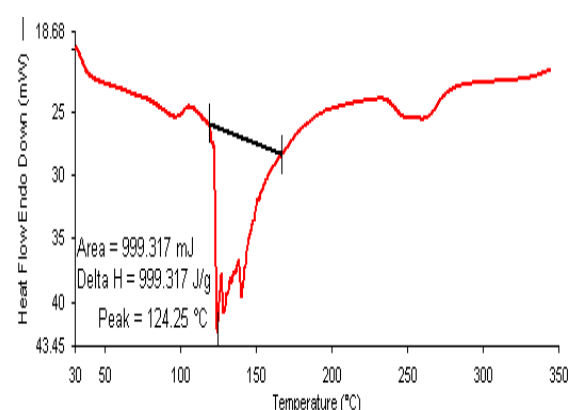
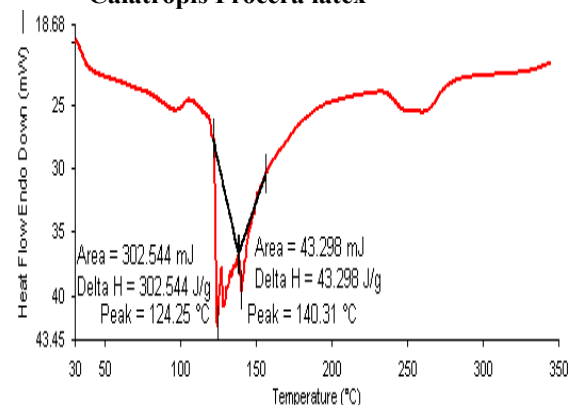


Figure 3 DSC for *Calatropis procera* latex

The Differential Scanning Calorimetry (DSC) thermograms show the thermal behaviour of a sample heated from 30 °C to 350 °C, revealing different aspects of the crystalline structure and phase transitions. Overall, both profiles show a large, sharp melting transition at 124.25 °C and a smaller melting transition at 140.31 °C ($\Delta H = 43.298 \text{ J/g}$, area = 43.298 mJ), suggesting either a mixed crystalline form or polymorphism. On the other hand, Figure 2 shows a single high-energy melting event at the same baseline (124.25 °C), with a considerably larger enthalpy change ($\Delta H = 999.317 \text{ J/g}$ or area = 999.317 mJ), which reflects a pure material with a very well-formed crystal lattice and without polymorphic transition. There are no exothermic peaks up to 350 °C, indicating that the sample does not crystallise or thermally degrade in this temperature range.

1.7 Total Phenolic Content

Gallic acid was used as the standard reference in the Folin–Ciocalteu colourimetry method to assess the Total Phenolic Content (TPC) of *Calatropis Procera* latex. The TPC was $14.68 \pm 0.42 \text{ mg}$ of gallic acid equivalents in the dry latex extract. By reducing oxidative degradation, phenolic compounds may improve the stability of the microemulsion formulation and enhance the latex's antioxidant capacity. These phenolic components may also enhance the formulation's antifungal activity and

boost its therapeutic efficacy against fungi that cause onychomycosis.

1.8 LCMS (Liquid Chromatography Mass Spectrometry):

The chromatogram showed a very strong peak at RT 18.38 min corresponding to the major UV-absorbing compound in the extract. The closely eluting constituents are indicated by additional peaks at 16.30, 16.66, 18.08 and 18.95 min. The base molecular feature in the corresponding ESI-MS spectrum is m/z 573.17, with ions at m/z 531.08, 709.08 and a number of higher-mass features. The major UV peak is shown to be related to a high molecular weight molecular feature and further confirms the chemical complexity of the extract.

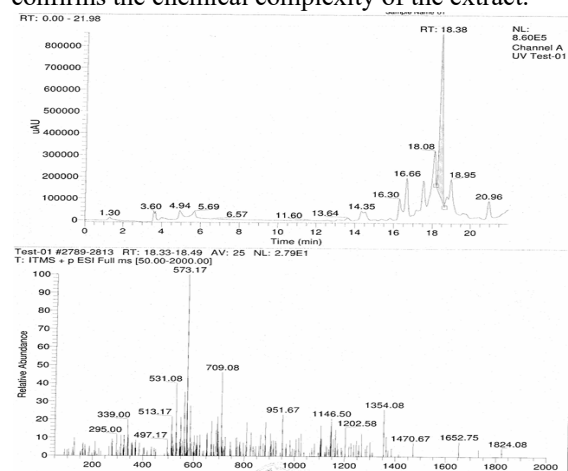


Figure 4 LC-MS chromatogram spectrum at RT 18.38

A unique signal at the chromatographic peak was seen at RT 20.95-20.96 min. The spectrum of its m/z showed an ion peak of 570.08 as the largest peak, followed by peaks at m/z 602.00, 552.17, 524.08 and 673.42, along with higher-mass ions. This profile shows that there is another structurally different molecular component or a molecular cluster that is eluting after the main peak at 18.38 min. This spectrum is also different in dominant m/z when compared to the 18.38-min spectrum, which further confirms there is compositional heterogeneity in the extract.

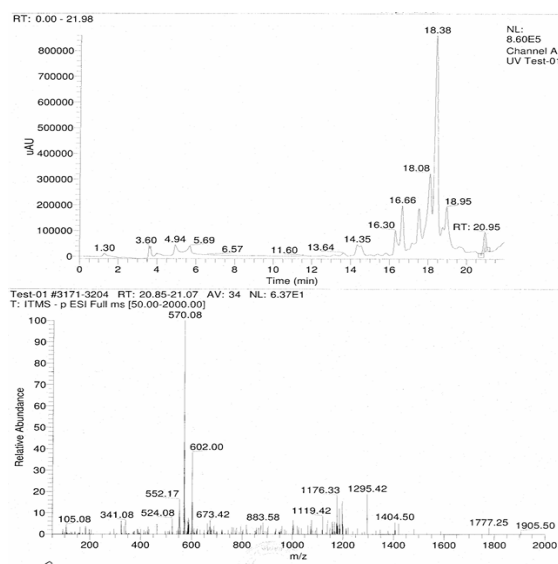


Figure 5 LC-MS chromatogram spectra at RT 20.95 min

A large peak was observed at around 18.95 min, adjacent to the large 18.38 min peak in the chromatogram. The mass spectrum showed m/z 515.17 as the base peak, m/z 668.25, and several molecular features at higher m/z values. The presence of a distinct dominant molecular ion at this retention time implies that the strong UV region is composed of more than one molecule, but that there are different chemical types.

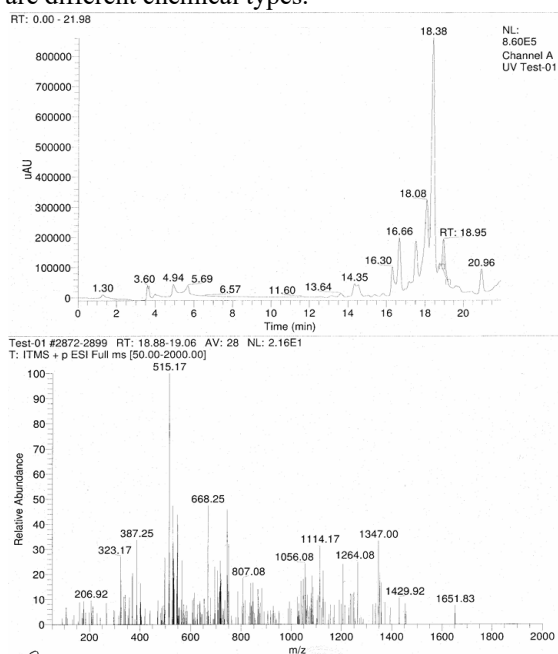


Figure 6 LC-MS chromatogram with mass spectra at RT 18.95

The chromatographic profile confirms reproducible detection of multiple constituents over the complete analytical run. The most prominent response occurred in the 16–20 min region, particularly at RT 18.08, 18.38 and 18.95 min, whereas several lower-intensity peaks occurred during the early and intermediate portions of the chromatogram. This distribution indicates that the extract contains both

relatively polar early-eluting compounds and comparatively less-polar/higher-retention constituents. The overall pattern is compatible with the complex secondary-metabolite composition reported for *C. procera*.

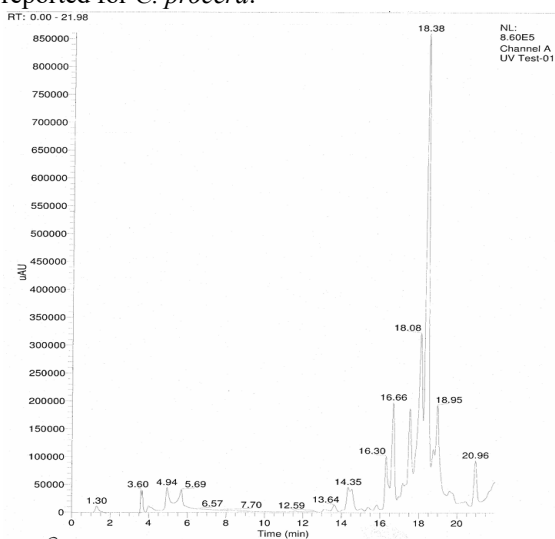
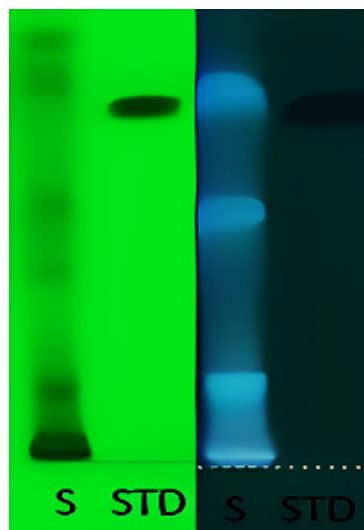


Figure 7 LC-MS chromatographic profile
1.9 HPTLC Analysis of Plant Latex Sample
1.9.1 Methodology

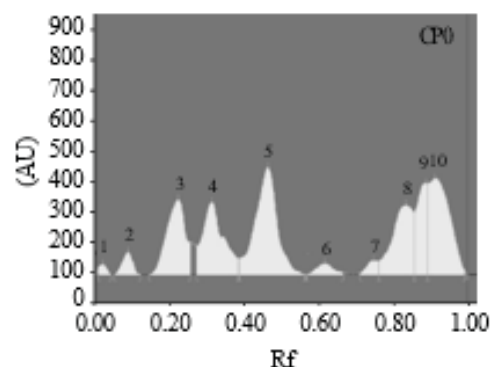
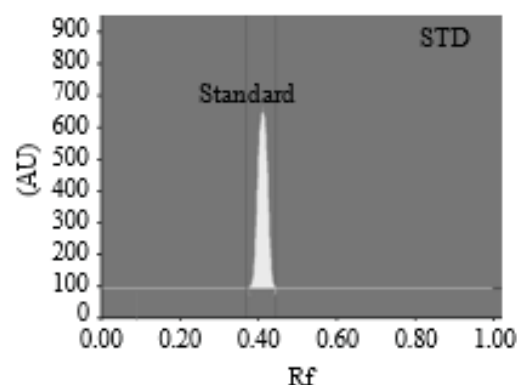
- 1. Sample Preparation:** The latex sample was collected, shade-dried, and dissolved in methanol to make a 2mg/ml solution. The solution was filtered through Whatman No. 1 filter paper and kept in an airtight vial for further analysis.
- 2. Preparation of Standard Solution:** Chelidone standard was prepared by dissolving it in methanol at a concentration of 1 mg/ml.
- 3. Chromatographic Conditions:** All pre-coated silica gel 60 F254 HPTLC plates (Merck) were used as the stationary Phase. Mobile Phase: Toluene: Ethyl acetate: Diethylamine (7:2:1). SAT: 20 minutes. Sample Application: Using a CAMAG Linomat 5 applicator, 2- 10 µL of both the standard and latex sample solutions were applied as bands of 6 mm. Development distance: 80 mm in a twin-trough chamber.
- 4. Detection and Visualization:** The developed plates were dried and visualized under UV light at 254 nm and 366 nm using a CAMAG UV-TLC viewer. After derivatization, the plates were observed under visible light to detect coloured spots.
- 5. Scanning and Densitometric Analysis:** Plates were scanned with a CAMAG TLC Scanner 4 at 254 nm. Chromatograms were recorded, and Rf values were calculated.

1.9.2 Calculation of Rf Values

$$R_f = \frac{\text{Distance traveled by the sample}}{\text{Distance traveled by the solvent front}}$$

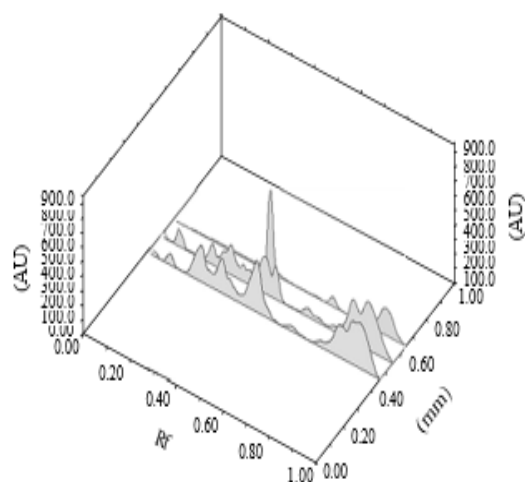


Multiple spots were observed on the developed plate under UV light, indicating the presence of various phytoconstituents in the latex sample.



The standard chelidone spot had an Rf value of approximately 0.43, corresponding to a sharp peak in the standard chromatogram.

The latex sample chromatogram showed multiple peaks with distinct Rf values, one of which matched the standard chelidone Rf value.



Track	Peak	Rf	Height	Area	Assigned Substance
CP01	1	0.02	29.4	412.4	Unknown
	2	0.08	74.5	2201.4	Unknown
	3	0.20	221.4	11417.3	Unknown
	4	0.32	223.0	3414.1	Unknown
	5	0.43	742.8	20500.1	Chelidonine
	6	0.67	32.5	1044.9	Unknown
	7	0.79	40.1	25474.1	Unknown
	8	0.81	225.0	1109.3	Unknown
	9	0.87	320.7	1526.2	Unknown
	10	0.90	315.0	12446.4	Unknown
Standard	1	0.42	571.8	15450.9	Chelidonine

The provided 3D densitogram illustrates the separation profile of phytoconstituents present in the plant latex sample

1.9.2.1 Rf Axis

The Rf values along the horizontal axis indicate the relative migration of different compounds on the TLC plate. Peaks at different Rf values suggest the presence of multiple phytoconstituents.

1.9.2.2 Peak Height (AU axis)

The height of each peak (measured in Absorbance Units, AU) represents the concentration or abundance of each compound. Higher peaks correspond to higher quantities of those compounds. A major, sharp peak is observed around Rf = 0.43, which correlates with the standard chelidonine reference peak, confirming its presence in the latex sample. Several other peaks of varying intensities are present, indicating a complex mixture of secondary metabolites in the latex extract. The smaller peaks at different Rf values demonstrate the presence of additional bioactive compounds apart from chelidonine.

Sample Chromatogram:

- Number of peaks observed: 10
- Major peak corresponding to chelidonine at Rf = 0.43
- Peaks were recorded with absorbance between 200–800 AU.

1.9.2.3 Standard Chromatogram

A single peak was observed at Rf = 0.43, corresponding to chelidonine with absorbance around 850 AU. The HPTLC analysis of the plant latex sample confirmed the presence of chelidonine by matching the Rf value and peak characteristics with the standard. In addition to chelidonine, several other phytoconstituents were present, as indicated by multiple peaks in the chromatogram. The method employed was effective for qualitative phytochemical profiling of the latex extract, showing good resolution and reliable detection under optimized conditions.

Ex vivo transgual permeation study using bovine/porcine hoof membrane:

The hydrated hoof membrane was placed between the donor and the receptor compartment, with the upper surface (dorsal) facing the donor compartment. To ensure "sink" conditions, the receptor compartment was filled with 20% methanol in phosphate buffer (pH 7.4) to a volume of 12 mL. The temperature of the receptor medium was kept at $32 \pm 0.5^\circ\text{C}$, the temperature of the nail bed, and the receptor medium was stirred continuously using a magnetic bead at 600 rpm. The optimized microemulsion (1 g) was applied in a uniform manner in the donor compartment. Aliquots (1 mL) were removed from the receptor compartment at regular time intervals (1, 2, 4, 6, 8, 12 and 24 hours) and replaced with an equivalent volume of fresh receptor medium. The samples collected were filtered through a membrane filter of $0.22\mu\text{m}$ mesh and spectrophotometrically analysed at 225nm.



Figure 8 Ex Vivo study using bovine/Porcine Hoof Membrane during lab perform.

The ex vivo transungual permeation performance of the optimized efinaconazole-loaded microemulsion was investigated using a Franz diffusion cell with hydrated bovine/porcine hoof membrane as a biomimetic model of the human nail plate. The experimental arrangement and membrane preparation are shown in the respective photographs. The hoof membrane was successfully mounted between the donor and receptor compartments with the dorsal keratinized surface oriented toward the formulation. The receptor compartment contained 12 mL of phosphate buffer pH 7.4 containing 20% methanol and was continuously stirred at 600 rpm at 32 ± 0.5 °C. These conditions were selected to maintain sink conditions and provide a physiologically relevant temperature for transungual delivery. The cumulative permeation of efinaconazole increased progressively with time, indicating continuous transport of the drug across the keratinized hoof membrane. The absorbance values obtained at 225 nm were 0.102, 0.214, 0.328, 0.437, 0.556 and 0.702 at the respective sampling intervals. Using the previously established calibration relationship for efinaconazole, the corresponding drug concentrations in the receptor medium were approximately 1.84, 3.38, 4.95, 6.45, 8.08 and 10.09 µg/mL, respectively. 1 mL receptor samples were withdrawn and immediately replaced with an equal volume of fresh receptor medium; cumulative drug permeation was corrected for sampling losses. The corrected cumulative amounts of efinaconazole permeated through the membrane

were approximately 22.03, 42.36, 64.56, 87.50, 113.59 and 145.78 µg at the six reported sampling points, respectively.

Table 1 Ex vivo transungual permeation profile of optimized efinaconazole microemulsion

Samp ling point	Absor bance at 225 nm	Calcu lated concent ration (µg/mL)	Corre cted cumul ative amou nt perme ated (µg)	Cumul ative drug perme ated* (%)
1	0.102	1.84	22.03	0.22
2	0.214	3.38	42.36	0.42
4	0.328	4.95	64.56	0.65
6	0.437	6.45	87.50	0.88
8	0.556	8.08	113.59	1.14
12	0.702	10.09	145.78	1.46
24	—	—	—	—

Calculated against 10 mg of efinaconazole present in 1 g of a 1% w/w formulation; the 24-h value cannot be calculated because the corresponding absorbance was not provided. The cumulative amount permeated increased approximately 6.6-fold between the first and last reported sampling points, from 22.03 µg to 145.78 µg. The approximately linear increase in receptor-phase drug concentration suggests sustained transport across the keratinized membrane rather than an abrupt burst of drug passage. Such behaviour is desirable for topical treatment of onychomycosis because prolonged drug availability at the nail barrier may improve the opportunity for drug deposition within the nail plate. The relatively low percentage of total applied drug detected in the receptor phase should not necessarily be interpreted as poor transungual performance. The nail plate is a dense keratinized barrier, and effective topical treatment requires substantial drug interaction with and retention within the nail rather than unrestricted passage into the receptor compartment. Previous investigations have shown that porcine hoof drug loading can correlate strongly with drug loading in human nail plates, although the

correlation for drug appearing in the receptor compartment is weaker.

DISCUSSION

The phytochemical characterization of the *Calotropis procera* extract demonstrated a chemically diverse profile, as evidenced by the complementary HPTLC and LC–MS analyses. The total phenolic content was determined to be **14.68 ± 0.42 mg gallic acid equivalents (GAE)/g dry extract**, indicating the measurable presence of phenolic constituents in the investigated extract[26]. HPTLC analysis further revealed a complex chromatographic fingerprint with multiple phytochemical bands, including a prominent band at **Rf 0.43**, which corresponded to the migration value of the chelidonine reference standard under the applied chromatographic conditions; however, this chromatographic correspondence should be considered supportive rather than definitive structural confirmation in the absence of orthogonal analytical confirmation[27]. The LC–MS chromatographic profile demonstrated several distinct molecular features, particularly in the 16–20 min retention-time region, with prominent signals at RT 18.38 min (*m/z* 573.17), RT 20.95 min (*m/z* 570.08), and RT 18.95 min (*m/z* 515.17). The feature at 18.38 min represented a major response in the chromatogram, with additional ions including *m/z* 531.08 and 709.08, whereas the 20.95-min feature was dominated by *m/z* 570.08 together with signals at *m/z* 602.00, 552.17, 524.08 and 673.42. A further prominent chromatographic feature at 18.95 min showed *m/z* 515.17 as the dominant ion, accompanied by a higher-mass feature at *m/z* 668.25[28,29]. The differences in the dominant mass-to-charge ratios and retention times indicate compositional heterogeneity within the extract and support the presence of multiple molecular constituents rather than a single dominant compound[30,31]. The overall LC–MS pattern, together with the HPTLC fingerprint and measurable phenolic content, is consistent with the complex secondary-metabolite composition previously described for *C. procera*. The observed phenolic content provides a quantitative chemical parameter for extract characterization and standardization and may contribute to the antioxidant-associated properties of the extract [32,33]; the present TPC and LC–MS data alone do not establish that individual phenolic or other detected metabolites are responsible for antioxidant activity. Similarly, the detected LC–MS features should be regarded as molecular features requiring further structural confirmation unless supported by authentic standards and/or[34,35] diagnostic MS/MS fragmentation. Collectively, the results establish a reproducible preliminary chemical profile of the investigated *C. procera* extract and provide an analytical basis for subsequent metabolite isolation, LC–MS/MS confirmation,

quantitative characterization and bioactivity-guided investigations[36].

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