RESEARCH PAPER

In Vitro Evaluation of Medicinal Plant Extracts Against *Trichophyton rubrum* and *Trichophyton mentagrophytes*

Sushma H. N.[1], Mrinalini Devi U.G.[2], Dr. T. Sharmila[1*]

[1] Department of Microbiology, Biotechnology and Food Technology, Bangalore University, Bengaluru 560056, Karnataka, India
[2] Department of Microbiology, Biotechnology and Food Technology, Bangalore University, Bengaluru 560056, Karnataka, India

**Corresponding author:* sushmahn1993@gmail.com

Abstract:

Background:

Medicinal plants such as *Piper betle*, *Curcuma longa*, *Trigonella foenum-graecum*, *Piper longum*, and *Mangifera indica* have a long history of use in Ayurveda and folk medicine for treating wounds, skin infections, and inflammatory conditions. In rural communities, these plants are commonly prepared as pastes, extracts, or decoctions for managing fungal infections. Despite this widespread traditional usage, there remains a scarcity of scientific evidence demonstrating their antifungal efficacy specifically against dermatophytic fungi.

Aim and Objectives:

The present study aimed to evaluate, under in vitro conditions, the antifungal activity of selected medicinal plant extracts against two clinically significant dermatophytes, *Trichophyton rubrum* and *Trichophyton mentagrophytes*, and to determine the minimum inhibitory concentrations of the most promising extracts.

Materials and Methods:

Ethanolic and methanolic extracts of *Trigonella foenum-graecum*, *Piper betle*, *Curcuma longa*, *Piper longum*, and *Mangifera indica* were prepared using Soxhlet extraction and tested for antifungal activity using the poisoned food technique. The minimum inhibitory concentration (MIC) was determined by the broth microdilution method, phytochemical screening, SEM, Column chromatography, TLC, Agar-based overlay technique bioautography, GC-MS and Cytotoxicity analysis was carried out using standard qualitative tests.

Results:

Among the 14 plant extracts screened, the ethanolic extract of *Piper betle* exhibited the highest antifungal activity, showing maximum mycelial growth inhibition of 95.2% against *T. rubrum* and 99.1% against *T. mentagrophytes* at 20 mg/mL. The lowest inhibition was recorded for *Senegalia ruguata* (13.75%) against *T. mentagrophytes* and *Nerium oleander* (26.05%) against *T. rubrum*. *Curcuma longa* ethanol extract displayed the lowest MIC value of 0.312 mg/mL against *T. rubrum*, while *Piper longum* and *Mangifera indica* methanol extracts showed a MIC of 0.312 mg/mL against *T. mentagrophytes.* Ethanolic extract of *Piper betle* was chosen for further phytochemical characterization based on better antifungal activity. Column chromatographic separation resulted in several fractions, of which fractions F4 and F5 exhibited detectable antifungal activity in TLC-bioautography, and active bands were observed at Rf values of about 0.70 and 0.79, respectively. SEM examination of treated dermatophytes showed clear morphological changes, such as hyphal deformation, surface shrinkage, and destruction of the mycelial architecture, compared to the untreated controls. The GC–MS analysis of the active extract showed several phytoconstituents that may be responsible for the observed antifungal activity. The cytotoxicity test in human keratinocyte cells (HaCaT) revealed a dose-dependent cytotoxicity with an IC_{50} value of 60.5µg/mL.

Conclusion:

The selected herbal extracts possess notable antifungal properties against dermatophytes and could serve as potential sources of natural antifungal agents. The phytochemical analysis revealed the presence of alkaloids, tannins, flavonoids, phenols, saponins, glycosides, and terpenoids. Fractions F4 and F5 were shown to be the most active by bioassay-guided fractionation, while hydroxychavicol and eugenol were found to be the main bioactive components by GC-MS analysis. Cytotoxicity analysis revealed satisfactory biocompatibility within the effective concentration range, while SEM examination verified significant ultrastructural damage to fungal hyphae, which may contribute to the observed antifungal effects.

Keywords: Minimum inhibitory concentration, *Trichophyton* spp., poisoned food technique, *Trigonella foenum-graecum*, *Piper betle*, *Curcuma longa*, *Piper longum*, *Mangifera indica*, cytotoxicity analysis.

How to cite this article: Sushma HN, Devi UGM, Sharmila T. In Vitro Evaluation of Medicinal Plant Extracts Against *Trichophyton rubrum* and *Trichophyton mentagrophytes*. Int J Drug Deliv Technol. 2026;16(89s): 1979-1997; DOI: 10.25258/ijddt.16.89s.186

Introduction

Dermatophytosis refers to superficial fungal infections of the skin, hair, and nails caused by a group of keratinophilic fungi known as dermatophytes. These fungi belong to three genera—*Trichophyton*, *Microsporum*, and *Epidermophyton*—and they colonise keratinised tissues such as the stratum corneum, nails, and hair shafts. Infection typically occurs through direct contact with infected individuals, animals, or contaminated fomites, including shared personal items and surfaces in communal facilities such as swimming

pools and barbershops [1]. The prevalence of dermatophyte infections is high worldwide, with tinea pedis (athlete's foot) being one of the most commonly encountered clinical presentations, particularly among adults aged 20 to 50 years [2].

The clinical management of dermatophytosis relies largely on synthetic antifungal agents classified into four major groups: polyenes, azoles, echinocandins, and the pyrimidine analogue 5-flucytosine. While these drugs have been the mainstay of treatment for decades, their long-term effectiveness is increasingly compromised by several factors. Fungal resistance to azoles has been reported with growing frequency, and treatment failures are not uncommon, particularly in chronic or recurrent infections. The pharmacological limitations of these agents—including adverse drug interactions, hepatotoxicity, and high treatment costs—further restrict their clinical utility, especially in resource-limited settings [3]. The emergence of antifungal resistance has been recognized as a serious public health concern, and the United States Centers for Disease Control and Prevention (CDC) has included drug-resistant fungal infections among its list of urgent antimicrobial resistance threats [4].

These challenges have renewed scientific interest in exploring plant-derived compounds as alternative or complementary antifungal therapeutics. Medicinal plants have been an integral component of traditional healthcare systems across the world for centuries, and their ethnopharmacological relevance is well documented. The World Health Organization (WHO) has estimated that a substantial proportion of the population in developing countries continues to rely on plant-based remedies for primary healthcare, with medicinal plants serving as the source of a considerable number of commercially available drugs [5]. In India, the Ayurvedic tradition prescribes numerous plant species for the treatment of skin ailments and fungal infections, though many of these traditional applications have not been subjected to rigorous scientific evaluation.

The antifungal properties of medicinal plants are largely attributed to their secondary metabolites, which include alkaloids, flavonoids, terpenoids, phenolic compounds, tannins, saponins, and glycosides. These phytochemicals exert their antifungal effects through various mechanisms, such as disrupting fungal cell membrane integrity, inhibiting ergosterol biosynthesis, interfering with enzyme activity, and generating oxidative stress within fungal cells [6,7]. Previous investigations have reported promising antifungal activity from a range of plant species. For instance, Abirami et al. [8] demonstrated the antifungal potential of acetone extracts from Indian medicinal plants against human dermal fungal pathogens, while Kedia et al. [9] documented the efficacy of essential oils against storage fungi and aflatoxin-producing strains.

Piper betle, commonly known as *betle* leaf, has been widely used in traditional medicine across South and Southeast Asia. Its leaves contain a range of bioactive compounds, including phenolic constituents such as hydroxychavicol, which have been associated with broad-spectrum antimicrobial activity. Similarly, *Curcuma longa* (turmeric) contains curcumin and related curcuminoids that possess documented anti-inflammatory and antimicrobial properties. *Trigonella foenum-graecum* (fenugreek) is rich in saponins and flavonoids, while *Piper longum* and *Mangifera indica* (mango) have also been reported to harbour bioactive constituents with antifungal potential [10,11].

Despite the growing body of literature on plant-derived antifungals, relatively few studies have systematically screened multiple medicinal plant extracts against the specific dermatophytes *T. rubrum* and *T. mentagrophytes* using standardised *in-vitro* protocols. The present study was therefore undertaken to bridge this gap by evaluating the antifungal activity of 14 medicinal plant extracts against these two clinically important dermatophyte species, identifying the most active extracts, determining their minimum inhibitory concentrations, and characterizing their phytochemical profiles. The antifungal activity of some medicinal plants against dermatophytes has been reported in previous studies. However, comprehensive studies involving antifungal screening combined with bioassay-guided-fractionation, phytochemical screening, mechanistic evaluation, and cytotoxicity assessment are still scarce. The present study is novel in that the selected medicinal plant extracts were systematically evaluated for their activity against *Trichophyton rubrum* and *Trichophyton mentagrophytes* by poisoned food and broth microdilution assays, chromatographic fractionation of the most active extract, TLC-based Agar-based overlay technique bioautography, GC-MS characterization of bioactive fractions, SEM analysis of fungal ultrastructural alterations and cytotoxicity evaluation on normal cells. The comprehensive method gives useful information on the antifungal potential, putative mechanism of action and preliminary safety profile of the identified bioactive ingredients supporting their prospective development as alternative treatment agents for dermatophytosis.

Dermatophytosis remains one of the most widespread superficial fungal diseases globally, and increasing antifungal resistance and treatment failure have led to an urgent need for safer and more effective therapeutic alternatives. Identification of bioactive phytochemicals that have significant antifungal activity and acceptable biocompatibility may help in the development of innovative plant-derived antifungal medicines for the therapy of dermatophyte infections. Therefore, the present study was designed to systematically investigate the antifungal potential of fourteen medicinal plant extracts against Trichophyton rubrum and Trichophyton mentagrophytes by agar well diffusion, poisoned food and broth microdilution (MIC) assays. The most active extract was submitted to bioassay-guided fractionation and GC–MS analysis of the active fractions. The antifungal mechanism was

studied by scanning electron microscopy (SEM) and the cytotoxicity of the active fractions was examined in HaCaT keratinocyte cells to assess their preliminary safety. This combination provided a holistic view of antifungal activity, bioactive ingredients, mechanism of action, and safety profile of *Piper betle* which supports its prospective development as a natural treatment agent for dermatophytosis.

Materials and Methods

Methanol, ethanol, ethyl acetate, and hexane were procured from Hi-Media Laboratories, Mumbai, India. Sabouraud's dextrose agar (SDA), Sabouraud's dextrose broth, ketoconazole, tetracycline, and RPMI-1640 liquid medium were also sourced from Hi-Media Laboratories. All chemicals used were of analytical grade.

Collection and Authentication of Plant Material

A total of 14 medicinal plants were selected for the study on the basis of a literature review and ethnobotanical survey. The plants, along with their accession numbers, were as follows: *Senegalia ruguata* DC (RRCBI-5736), *Azadirachta indica* A. (RRCBI-8970), *Trigonella foenum-graecum* L. (RRCBI-mus105), *Piper betle* L. (RRCBI-mus113), *Curcuma longa* L. (RRCBI-mus277), *Eucalyptus globulus* Labill. (RRCBI-17965), *Calotropis gigantea*Ait. (RRCBI-8770), *Piper longum* L. (RRCBI-mus454), *Mangifera indica* L. (RRCBI-mus110), *Syzygium cumini* L. (RRCBI-5418), *Plectranthus amboinicus*Lour. (RRCBI-mus214), *Senna siamea*L. (RRCBI-19156), *Nerium oleander* L. (RRCBI-15290), and *Ficus racemosa* L. (RRCBI-8021).

Leaves, rhizomes, seeds, and pods were collected from mature plants growing in the vicinity of Bangalore University, Jnana Bharathi Campus, and Gandhi Krishi Vigyana Kendra (GKVK), Bengaluru. All plant specimens were authenticated by Dr. V. Ramarao, Research Officer, Central Council for Research in Ayurvedic Sciences (CCRAS), Bengaluru 560109, India.

Preparation of Extracts

The collected plant parts were washed thoroughly with tap water two to three times, shade-dried at room temperature for 15 days, and ground into a fine powder using a mechanical grinder. The powdered material was stored in airtight containers until extraction.

Soxhlet Extraction

Extracts were prepared using a Soxhlet apparatus. Approximately 65 g of each powdered plant sample was placed in a thimble and extracted with the appropriate solvent at a plant material-to-solvent ratio of 1:3 (w/v). The extraction was carried out for 48 h using solvents of increasing polarity: hexane (45 °C), ethyl acetate (60 °C), and ethanol or methanol (75 °C). Following extraction, the solvents were removed by rotary evaporation, and the concentrated extracts were stored at 4–5 °C for subsequent use.

Extraction Yield

The percentage yield of each extract was calculated using the following formula:

$$Percentage\ yield\ (\%) = (Wa / Wb) \times 100$$

where Wa represents the weight of the crude extract obtained and W_b represents the weight of the dried plant powder taken for extraction.

Procurement and Maintenance of Fungal Cultures

Standard strains of *Trichophyton rubrum* (MTCC-3272) and *Trichophyton mentagrophytes* (MTCC-7687) were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC) at the CSIR-Institute of Microbial Technology, Chandigarh, India. The cultures were maintained on Sabouraud's dextrose agar (SDA) slants and sub-culture periodically. For each experiment, fresh cultures were prepared by inoculating the fungi onto SDA plates and incubating at room temperature (25 ± 2°C) for five to seven days.

Inoculum Preparation

Inoculum suspensions were prepared from fresh cultures (3 to 7 days old) grown on SDA plates. Sterile distilled water (5 mL) was added to each plate, and the surface of the colony was gently scraped with a sterile loop to release the spores. Two to three drops of Tween 80 were added to facilitate spore dispersion. The resulting suspension was filtered through sterile gauze to remove hyphal fragments and then centrifuged at 6000 rpm for 15 min. The pellet was resuspended in sterile distilled water, and the turbidity was adjusted to 0.5 McFarland standard in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. The inoculum suspensions were prepared following the M38-A2 protocol of the National Committee for Clinical Laboratory Standards (NCCLS, 2004) [12].

Preparation of plant extracts

Stock solutions of the selected medicinal plants were prepared by dissolving the dried crude extracts in 5% (v/v) dimethyl sulfoxide (DMSO). Aseptic solutions were obtained by filtering solutions using a 0.22 µm membrane filter (Millipore, USA). Stock solutions were added to the sterilized molten Sabouraud Dextrose Agar (SDA) kept at 45–50°C with appropriate quantities to obtain the needed final concentrations for the antifungal evaluation. The extracts were examined in the poisoned food assay at final concentrations of 10 and 20 mg/mL. Fresh extract solutions were made before to each experiment.

Vehicle control

A 5% (v/v) dimethyl sulfoxide (DMSO) solution served as the vehicle control. The solution was prepared by mixing 5 mL DMSO and 95 mL sterile distilled water (or sterile physiological saline/PBS, depending on the solvent used for the test extract) to a final concentration of 5% DMSO . The vehicle control did not contain plant extract and served as a control for any effect of the solvent alone on fungal growth or the experimental model. Vehicle controls were treated with an equivalent

volume of the 5% DMSO vehicle for the same experimental conditions as the treatment groups.

Antifungal Activity by Poisoned Food Technique

The poisoned food technique (PFT) was employed to evaluate the antifungal activity of the crude plant extracts. Each extract was incorporated into molten SDA medium at a concentration of 1 mg/mL, mixed thoroughly, and autoclaved. The medium (20 mL per plate) was poured into sterile Petri dishes and allowed to solidify. After solidification, 10 µl of the standardised spore suspension was placed at the centre of each agar plate. The experiment was conducted in triplicate. Control plates consisted of SDA medium without any extract. Ketoconazole (25 µg/mL) served as the positive control, and 5% dimethyl sulfoxide (DMSO) was used as the negative (vehicle) control. The plates were incubated at room temperature for five to seven days, after which the diameter of the fungal colony in each plate was measured. The percentage inhibition of mycelial growth was calculated using the formula:

$$\% \, Inhibition = [(dc - dt) / dc] \times 100$$

where *dc* is the mean colony diameter in the control plate and *dt* is the mean colony diameter in the treated plate.

Minimum Inhibitory Concentration by Broth microdilution Method

The minimum inhibitory concentration (MIC) of the selected plant extracts was determined using the broth microdilution method. Each extract was prepared at an initial concentration of 2.5 mg/mL and subjected to serial two-fold dilution in 5% DMSO to obtain concentrations ranging from 0.004 mg/mL to 2.5 mg/mL. Spore suspensions were prepared in RPMI-1640 medium and adjusted to 0.5 McFarland standard. Each well of a 96-well microtiter plate received 100 µl of RPMI-1640 medium, 100 µl of the herbal extract, and 20 µl of spore suspension. Ketoconazole at 25 µg/mL served as the positive control, and 5% DMSO served as the negative control. The plates were incubated at room temperature for 72 h. The MIC was determined as the lowest concentration of extract that showed no visible fungal growth, as assessed by visual inspection and comparison with the growth control, following the NCCLS M38-P guidelines [13].

Phytochemical screening

Test for phenols (lead acetate test): Three millilitres of the filtrate were treated with 1 mL of 10% lead acetate solution. The formation of a milky white precipitate confirmed the presence of phenols.

Test for saponins (foam test): The crude extract was mixed with 5 mL of distilled water in a test tube and shaken vigorously. The persistence of a stable foam layer was considered indicative of saponins.

Test for terpenoids (Salkowski test): Two millilitres of concentrated sulphuric acid were carefully added to the crude extract dissolved in chloroform. The development of a reddish-brown colouration at the interface confirmed the presence of terpenoids.

Test for glycosides (Liebermann's test): The crude extract was treated with 2 mL of acetic acid and 2 mL of chloroform. The mixture was cooled in ice, and concentrated sulphuric acid was added carefully along the side of the test tube. A colour change progressing from violet-blue through blue to green was taken as a positive result for glycosides.

Test for anthocyanins: Two millilitres of the plant extract were mixed with 2 mL of 2N hydrochloric acid. A pinkish-red colouration that turned bluish-violet upon the addition of ammonium hydroxide indicated the presence of anthocyanins.

Scanning electron Microscopy

Scanning electron microscopy (SEM) was performed to investigate the effect of the ethanolic extract of *Piper betle* leaves on the morphology of *T.rubrum* and *T. mentagrophytes* described by (madzinga et.al), with slight modification. Fungal agar blocks (55 mm) control and agar blocks of mycelium from the treated plates were thinly cut by using the forceps and needles and fixed using a fixative solution containing 2.5%(v/v) glutaraldehyde and 5% (v/v) formaldehyde in 0.075M phosphate buffer and incubated for 8 hrs at 4^0 C. Thereafter, the mycelium was washed thrice with the phosphate buffer (15 min for each wash) and dehydrated using a gradient series of ethanol solutions (30%, 50%, 70%, 90%, 95%, and 3100 %) for 15 min for each series. The materials were then left in 100% ethanol for 7 days, followed by critical point. Dried samples were mounted on aluminum stubs lined with conductive material and coated with gold using a sputter coater (Model Evo 10, Carl Zeiss make, Germany) operating at 5.00 kV. Images were taken at 1.00 KX magnification.

Column Chromatographic Fractionation of Ethanolic Extract of *Piper betle* Leaves

Using silica gel (60–120 mesh) as the stationary phase, the ethanolic extract of *Piper betle* leaves was separated by column chromatography. Using chloroform as the packing solvent, a glass column was packed using the wet packing technique. The concentrated ethanolic extract was carefully put into the top of the packed column after being adsorbed onto a small amount of silica gel and dried. Chloroform:ethyl acetate (7:3, v/v) was the solvent system used for elution. Fractions were collected in clean, labeled collection tubes one after the other while the eluent was permitted to run through the column at a steady rate. The chromatographic separation produced a total of six fractions (F1–F6).

Thin Layer Chromatography (TLC) on silica gel 60 F254 plates was used to monitor the collected fractions under reduced pressure using a rotary evaporator and the same solvent system (chloroform: ethyl acetate, 7:3 v/v). When necessary, a 1% vanillin reagent was sprayed on the produced TLC plates to further detect spots. The plates were viewed under UV light at 254

and 366 nm. For additional agar lay bioautography, GC-MS, and cytotoxicity investigations, fractions with comparable TLC patterns were combined and concentrated.

Agar-based overlay technique Bioautography of Column Fractions

The Agar-based overlay technique bioautography method was used to assess the antifungal activity of the six fractions (F1–F6) that were generated from column chromatographic separation of the ethanolic extract of *Piper betle* leaves. Each fraction was put as distinct bands onto silica gel 60 F254 TLC plates after being dissolved in ethanol at the proper concentration. Chloroform: ethyl acetate (7:3, v/v) was used as the mobile phase to develop the plates, and any remaining solvent was eliminated by letting the plates air dry completely.

The TLC plates were aseptically covered with molten SDA media that had already been inoculated with the test microorganisms (*T. rubrum* and T. mentagrophytes) at about 10^6 CFU/mL after chromatographic development. After allowing the agar-coated plates to harden, they were incubated for five to seven days at room temperature. Following incubation, zones of inhibition became visible, signifying the presence of substances having antifungal activity within the chromatographic bands.

GC–MS Analysis of Bioactive Fractions

Gas Chromatography–Mass Spectrometry (GC–MS) analysis was performed for phytochemical characterization of the bioactive fractions F5 and F6, which were obtained from column chromatographic separation of the ethanolic extract of *Piper betle* leaves and showed the highest antifungal activity in Agar-based overlay technique bioautography.

A Shimadzu GCMS-QP2010 system with an electron ionization (EI) mass spectrometer and a GC-2010 gas chromatograph was used for GC–MS analysis. An HP-5 capillary column (30 m × 0.32 mm i.d., 0.25 µm film thickness) was used to achieve separation. The carrier gas was 99.999%purehelium. The fractions were filtered through a 0.22 µm membrane filter and concentrated under low pressure before analysis. An AOC-20i autosampler operating in split mode was used to inject an aliquot of 1 µL of each sample through a split/split less injector kept at 250°C. In accordance with the instrument specifications, the carrier gas flow and inlet conditions were optimized. The temperature program for the GC oven was set to start at 60°C, hold it for two minutes, raise it to 280°C at a rate of 10°C min^{-1}, and then keep it there for ten minutes. The temperature of the transfer line was kept at 280°C.

At 70 eV, mass spectral detection was performed in electron ionization (EI) mode. Mass spectra were acquired across a scan range of m/z 50–600 while the ion source temperature was kept at 200°C. Shimadzu GCMS software was used for data collection and processing.

By comparing their mass spectra with those found in the National Institute of Standards and Technology (NIST) Mass Spectral Library, the phytochemical components found in fractions F5 and F6 were identified. Retention times, molecular weights, molecular formula, and relative peak area percentages were used to characterize the discovered compounds. The found antifungal activity of the fractions was connected with compounds having greater peak area percentages, which were regarded as the primary constituents.

Cytotoxicity assay

The ethanolic extract of piper betle leaves was subjected to cytotoxicity analysis against HaCaT (human keratinocyte cell line) and was purchased from NCCS, Pune, India. The cells were maintained in DMEM high glucose media supplemented with 10% FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2 and 18-20% O_2 at 37^0C temperature in the CO_2 incubator and sub-culture every 2 days (Barros et.al). Seed 1000 µl cell suspension in a 12-well plate at the required cell density (50,000 cells per well), without the test agent. Allow the cells to grow for about 24 hours. Sterilize the given samples by keeping them under UV light for 30 mins and washing with 1x PBS 2 times to ensure sterility. After sterilization, carefully keep the samples in each well of the 12-well plate with the help of sterile forceps and replace wells with fresh culture medium. Cells without any sample were considered untreated, and cells treated with doxorubicin at a 1 µM/mL concentration was considered a positive control for the study. Incubate the plate for 48 hrs at 37°C in a 5% CO_2 atmosphere. After the incubation period, take out the plates from the incubator and remove spent media and add MTT reagent to a final concentration of 0.5 mg/mL of total volume. Wrap the plate with aluminum foil to avoid exposure to light. Return the plates to the incubator and incubate for 3 hours. (Note: Incubation time varies for different cell lines. Within one experiment, incubation time should be kept constant while making comparisons.) Remove the MTT reagent and then add 100µl of solubilization solution (DMSO). Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals, especially in dense cultures. Read the absorbance on a spectrophotometer or an ELISA reader at a 570 nm wavelength. (Alley *et al* and Mosamann *et al*).

% Cell viability is calculated using the below formula:

% cell viability = [Mean abs of treated cells/Mean abs of Untreated cells] x 100

Statistical Analysis

All experiments were carried out in triplicate, and the data were expressed as mean ± standard deviation. Statistical analysis was performed using the SPSS software package (version 20.0). The data were analysed by two-way analysis of variance (ANOVA) followed by Tukey's post hoc test to compare

differences among treatment groups. A p-value of less than 0.05 was considered statistically significant.

Results

Antifungal Activity of Crude Extracts Against T. mentagrophytes and T. rubrum

Of the 14 plant extracts screened, the ethanolic extract of *Piper betle* exhibited the strongest antifungal activity against both dermatophyte species. At a concentration of 20 mg/mL, the crude ethanolic extract of *Piper betle* produced 95.2 ± 2.17% inhibition of mycelial growth against *T. mentagrophytes*. The remaining extracts showed inhibition in the following descending order: *Trigonella foenum-graecum* (65.9 ± 1.0%), *Mangifera indica* (64.2 ± 3.0%), *Piper longum* (63.6 ± 2.8%), *Curcuma longa* (59.6 ± 0.6%), *Azadirachta indica* (53.9 ± 6.7%), *Eucalyptus globulus* (54.1 ± 0.4%), *Calotropis gigantea*(51.4 ± 1.8%), *Ficus racemosa* (34.0 ± 1.4%), *Syzygium cumini* (30.5 ± 4.3%), *Nerium oleander* (28.0 ± 7.9%), *Senna siamea*(27.1 ± 3.0%), *Plectranthus amboinicus*(26.0 ± 1.4%), and *Senegalia ruguata*(13.75 ± 1.76%). The positive control, ketoconazole at 25 µg/mL, showed 74.5% inhibition, The vehicle control (5% DMSO) showed no inhibition of fungal growth, indicating that the solvent used did not have antifungal action under the circumstances of the assay. At the lower concentration of 10 mg/mL, *Piper betle* also showed notable activity against *T.mentagrophytes*, with 79.95 ± 0.75% inhibition, while *Senegalia ruguata*recorded the lowest inhibition at 16.55 ± 7.70%.Against *T. rubrum* at 20 mg/mL, Piper betle demonstrated 99.1 ± 0.9% inhibition of mycelial growth, the highest among all the extracts tested. The lowest inhibition against *T. rubrum* was observed for *Nerium oleander* (26.05 ± 7.0%). The positive control (ketoconazole, 25 µg/mL) gave 76.5% inhibition, and vehicle control 5 % DMSO no percentage of inhibition. On the basis of these results, five plants—*Trigonella foenum-graecum*, *Piper betle*, *Curcuma longa*, *Piper longum*, and *Mangifera indica*—were selected for further investigation. *Piper betle* showed 95.2% inhibition against *T. rubrum* and 99.1% inhibition against *T. mentagrophytes* at the maximum tested concentration (20 mg/mL). Ketoconazole was used as the positive control under the same experimental circumstances and showed good antifungal efficacy at 25 µg/mL. These results show that the crude extract has a high antifungal capability; nevertheless, a direct quantitative comparison between the extract and ketoconazole is not suitable since they were evaluated at different concentration levels.

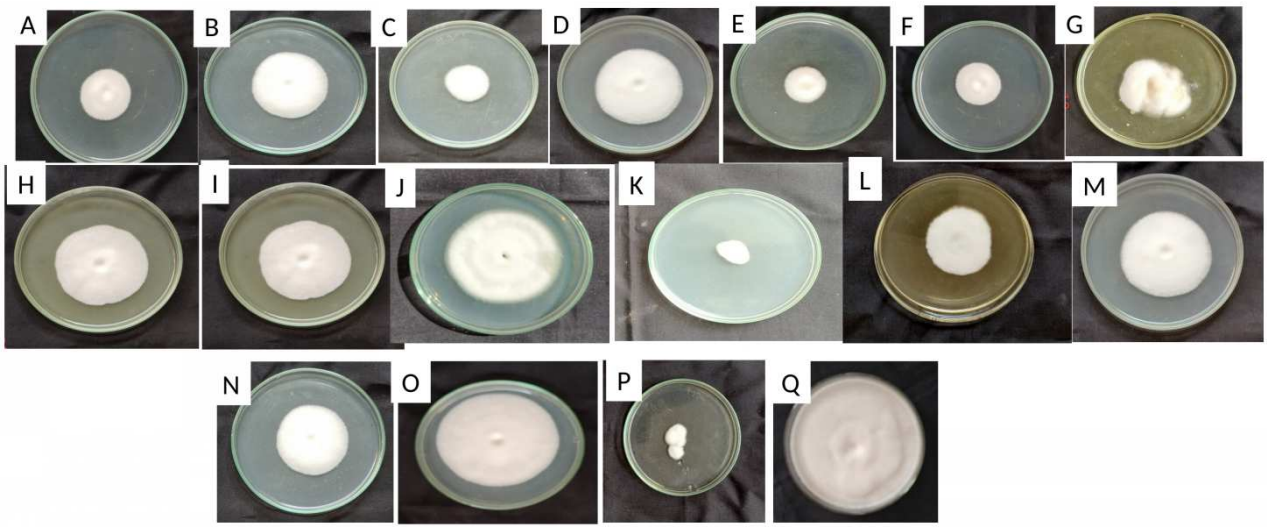


Figure 1. Antifungal activity of selected plant extracts at 20mg/ml concentration against the *T. rubrum* A- extract of *Eucalyptus globulus*, B- extract of *Calotropis gigantea*, C- extract of *Piper longum*, D- extract of *Azadirachta indica*, E- extract of *Trigonella foenum–graceum*, F- extract of *Mangifera indica*, G- extract of *Curcuma longa*, H- extract of *Coleus amboinicus*, I- extract of *Ficus racemosa*, J- extract of *Senegalia ruguata*, K- extract of *Piper betle*, L- extract of *Senna siamea*, M- extract of *Nerium oleander*, N- extract of *Syzygium cumini*, O- Vehicle control (5% DMSO), P- Positive control (25 µg/ml Ketoconazole), Q- Organism control.

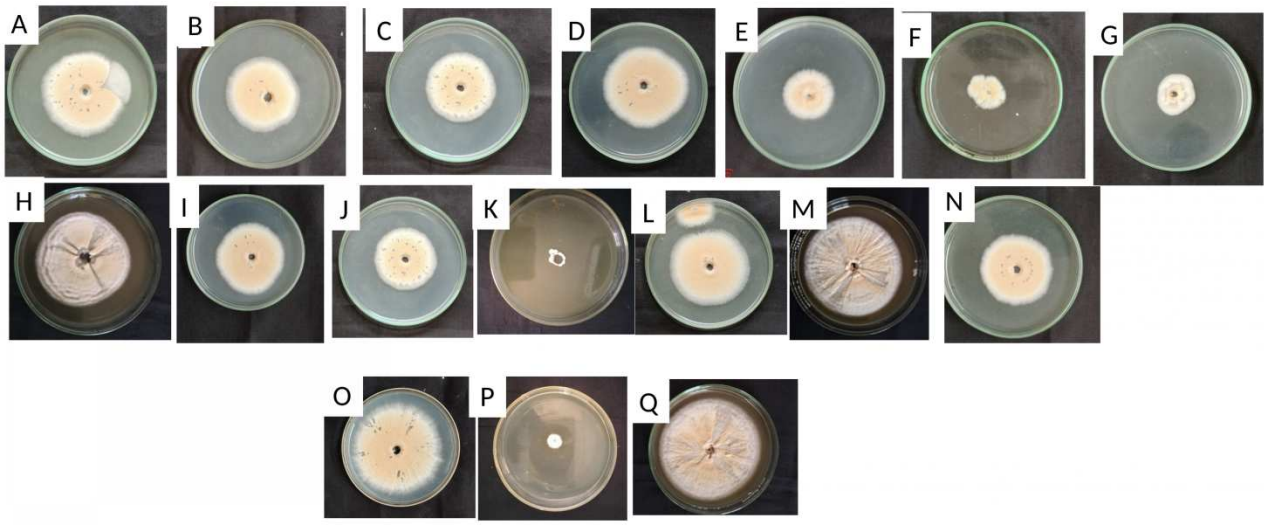


Figure 2. Antifungal activity of selected plant extracts at 20mg/ml concentration against the *T. mentagrophytes* A- extract of *Eucalyptus globulus*, B- extract of *Calotropis gigantea*, C- extract of *Piper longum*, D- extract of *Azadirachta indica*, E- extract of *Trigonella foenum–graceum*, F- extract of *Mangifera indica*, G- extract of *Curcuma longa*, H- extract of *Coleus amboinicus*, I- extract of *Ficus racemosa*, J- extract of *Senegalia ruguata*, K- extract of *Piper betle*, L- extract of *Senna siamea*, M- extract of *Nerium oleander*, N- extract of *Syzygium cumini*, O- Vehicle control (5% DMSO), P- Positive control (25 µg/ml Ketoconazole), Q- Organism control.

Table 1. Antifungal activity of the plant extracts against *Trichophyton mentagrophytes*

		% inhibition of Mycelial growth
Sl no	**Botanical name**	**20mg/ml**
1	*Acacia concina.DC*	23.5±1.4
2	*Azadirachta indica A.*	53.9±6.7
3	***Trigonella foenum- graceum L.***	**65.9±1***
4	***Piper betle L.***	**95.2±2.1***
5	*Curcuma longa L.*	59.6±0.6
6	*Eucalyptus globulus Labill.*	54.1±0.4.
7	*Calotropis procera Ait.*	51.4±1.8
8	***Piper tubercutalum L.***	**63.6±2.8***
9	***Mangifera indica MI.***	**64.2±3***
10	*Syzygium cumini L*	30.5±4.3
11	*Coleus amboinicus Lour*	26±1.4
12	*Nerium oleander L*	28±7.9
13	*Cassia fistula L*	27.1±3.0
14	*Ficus racemosa L*	34±1.4

Data given are mean of triplicates (n=3) ± standard error (SE). Different lower case letters (*) in the same column indicate significant differences according to ANOVA and Tukey's multiple tests (P<0.05).

Table 2. Antifungal activity of the plant extracts against *Trichophyton rubrum*

		% inhibition of Mycelial growth
Sl no	**Botanical name**	**20mg/ml**
1	*Acacia concina.DC*	41.2±4.5
2	*Azadirachta indica A.*	54.8±4.8
3	***Trigonella foenum -graceum L.***	**68.3±7.5***
4	***Piper betle L.***	**99.1±0.9***
5	***Curcuma longa L.***	**78.45±3.5***
6	*Eucalyptus globulus Labill.*	64.6±3.5
7	*Calotropis procera Ait.*	60±3.9
8	*Piper tubercutalum L.*	69.5±4.7
9	***Mangifera indica MI.***	**70.1±5.4***
10	*Syzygium cumini L*	31.8±14.3
11	*Coleus amboinicus Lour*	27±1.4
12	*Nerium oleander L*	25.85±5.8
13	*Cassia fistula L*	28.3±16.5
14	*Ficus racemosa L*	29±1.4

Data given are mean of triplicates (n=3) ± standard error (SE). Different lower case letters (*) in the same column indicate significant differences according to ANOVA and Tukey's multiple tests (P<0.05).

Minimum Inhibitory Concentration (MIC) Against *Trichophyton rubrum* and *Trichophyton mentagrophytes*.

The MIC values of the five selected plant extracts against *T. rubrum* and *T. mentagrophytes* are summarised below. Against *T. rubrum*, *Curcuma longa* ethanol extract showed the strongest activity, with an MIC of 0.312 mg/mL. *Trigonella foenum-graecum* ethanol extract demonstrated an MIC of 1.25 mg/mL. The methanol and ethanol extracts of *Mangifera indica* and *Piper betle* exhibited an MIC of 5 mg/mL against this species.

Against *T. mentagrophytes*, the ethanol and methanol extracts of *Piper longum* and *Mangifera indica* displayed the strongest antifungal activity, each with an

MIC of 0.312 mg/mL. The ethanol extract of *Piper betle* showed an MIC of 0.625 mg/mL, while the ethanol extract of *Trigonella foenum-graecum* had an MIC of 1.25 mg/mL.

Table 3. Minimum inhibitory concentration (MIC) values of the selected plant extracts against *Trichophyton rubrum*

Sl no	Botanical name	Extract	MIC(mg/ml)
1	*Trigonella foenum-graceum L.*	Ethanol	1.25
2	*Piper betle L.*	Ethanol	5
3	*Curcuma longa L.*	Ethanol	0.312
4	*Mangifera indica MI.*	Methanol	5

Table 4. Minimum inhibitory concentration (MIC) values of the selected plant extracts against *Trichophyton mentagrophytes*

Sl no	Botanical name	Extract	MIC(mg/ml)
1	*Trigonella foenum-graecum L.*	Ethanol	1.25
2	*Piper betle L.*	Ethanol	0.625
3	*Piper tuberculatum.*	Ethanol	0.312
4	*Mangifera indica MI.*	Methanol	0.312

Phytochemical screening of Selected Crude Extracts

Qualitative phytochemical screening of the five selected plant extracts revealed the presence of diverse secondary metabolites. The methanol extract of *Trigonella foenum-graecum* was found to contain alkaloids, tannins, flavonoids, glycosides, and terpenoids. The methanol extract of *Mangifera indica* tested positive for phenols, terpenoids, alkaloids, tannins, and glycosides. *Piper longum* methanol extract contained alkaloids, tannins, flavonoids, phenols, saponins, glycosides, and terpenoids. The ethanol extract of *Piper betle* showed the presence of alkaloids, tannins, flavonoids, phenols, glycosides, and terpenoids. *Curcuma longa* extract was found to contain phenols, terpenoids, tannins, and alkaloids.

Analysis of Scanning Electron Microscopy(SEM)

To assess the ultrastructural alterations brought about by the bioactive portion of the ethanolic extract of *Piper betle* leaves on *Trichophyton rubrum* and *Trichophyton mentagrophytes*, scanning electron microscopy (SEM) was used. Normal fungal morphology was demonstrated by the untreated fungal cells (control), which had smooth, elongated tubular hyphae with intact cell walls, continuous mycelia, and consistent branching patterns. On the other hand, both dermatophytes showed significant morphological changes after being treated with the bioactive fraction of *Piper betle*. The smooth surface of the control hyphae was replaced by a rough and uneven surface in the treated hyphae. The fungal cell wall lost structural integrity and crumpled, with visible surface corrugations. Along with uneven branching and mycelial network distortion, the usual tubular hyphae seemed folded and deformed. Additionally, the treated mushrooms showed mycelium fragmentation, which suggests that the hyphal continuity was disrupted. Frequent observations of hyphal shrinkage, constriction, and rupture indicate significant damage to the fungal cell membrane. The hyphae revealed deep surface depressions and collapsed structures in some areas, while they appeared flattened, compressed, and fused together in others. In contrast to the normal spores seen in the untreated control, fungal spores appeared distorted and irregular in shape where they were present.

Trichophyton mentagrophytes was more susceptible to the *Piper betle* bioactive fraction than *Trichophyton rubrum*, as evidenced by the more noticeable morphological changes. The *Piper betle* extract significantly alters the shape of fungal cells, as evidenced by the considerable damage seen by SEM, which includes roughened hyphal surfaces, wrinkled cell walls, collapse of tubular structures, distortion of branching patterns, fragmentation of the mycelium, and hyphal shrinkage.

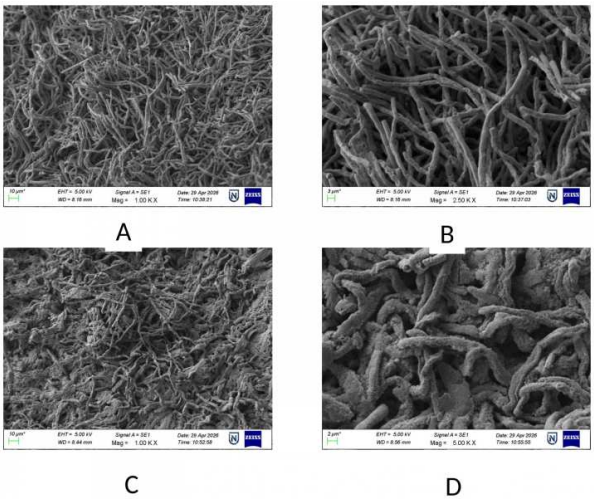


Figure 3. Scanning electron micrographs of *Trichophyton mentagrophytes* before and after treatment with the ethanolic extract of *Piper betle*. (A & B) *T. mentagrophytes* untreated control (mag-5.00k×&1.00kx); (C & D) Treated (mag-5.00k×&1.00kx) *T. mentagrophytes*. Treated fungi exhibited severe ultrastructural alterations, including surface roughening, cell wall wrinkling, hyphal collapse, distortion, fragmentation and shrinkage compared with the untreated controls.

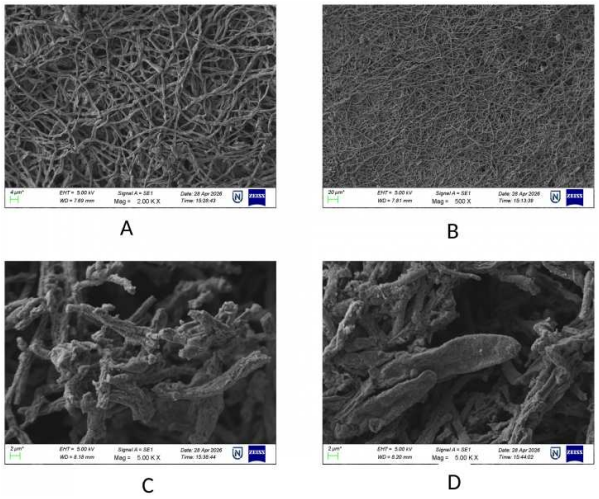


Figure 4. Scanning electron micrographs of *Trichophyton rubrum* before and after treatment with the ethanolic extract of *Piper betle*. (A & B) *T. rubrum* untreated (mag-5.00k×&1.00kx); (C & D) Treated (mag-5.00k×&1.00kx) *T. rubrum*. Treated fungi exhibited severe ultrastructural alterations, including surface roughening, cell wall wrinkling, hyphal collapse, distortion, fragmentation and shrinkage compared with the untreated controls.

Column Chromatography

The ethanolic extract of *Piper betle* leaves was submitted to column chromatography over silica gel using chloroform acetate (7:3, v/v) as solvent system. The elution of the column resulted in the separation of the crude extract into six different fractions F1, F2, F3, F4, F5 and F6. The fractions were collected according to the variation in band migration and elution characteristics in the chromatographic separation.

Thin layer chromatography

TLC was carried out for the six fractions (F1-F6) collected through column chromatography separation to isolate and identify the bioactive compounds of the piper betle leaf extract. Ethanolic extract of *Piper betle* leaves was run on thin layer chromatography (TLC) with a silica gel 60 F254 plates and chloroform: ethyl acetate (7:3, v/v) solvent system. The chromatograms exhibited good separation patterns within the fractions and satisfactory fractionation of the phytochemical contents. The Rf values of the spots in fractions F1, F2, F3, and F6 were considerably low which indicates the presence of more polar elements. The fractions F4 and F5 showed well-defined bright patches with higher Rf values, indicating enrichment of less polar bioactive chemicals. UV (254 and 366 nm) exposure resulted in variable fluorescence intensity and spot distribution amongst fractions. The TLC fingerprints of the different extracts confirmed the discovery of phytochemical components by column chromatography. The compounds having Rf values of 0.7 and 0.79 were visualized in F4 and F5 TLC chromatogram [26].

Table 7. Thin layer chromatography (TLC) profile of ethanolic extract of *Piper betle* leaf

Fractions	Rf value	Visual	UV-254nm	UV-366nm	1% Vanillin sulfuric acid spray
F-1	-	No spots	No spots	No spots	No spots
F-2	-	No spots	No spots	No spots	No spots
F-3	0.4	Light Yellow	green	yellow	Light yellow
F-4	0.7	Yellow	Dark green	Orange	Yellow
F-5	0.79	Light green	Dark green	Orange	Light yellow
F-6	0.3	White	light yellow	yellow	Light yellow

Agar over lay -Bioautography

The antifungal activity of the chromatographic fractions was tested by the Agar-based overlay technique bioautography method. Among examined fractions, Fraction F4 and Fraction F5 exhibit unique antifungal activity towards the tested dermatophytes, suggesting the presence of bioactive ingredients. Fraction F4 showed a good inhibition zone against *Trichophyton mentagrophytes* with a zone of inhibition of 16 mm showing excellent antifungal activity. The inhibitory zone was visualized as a clear zone against the stained backdrop, which was the bioactive band on the TLC plate[26,27]. Fraction F5 also showed antifungal activity against Trichophyton rubrum with a clear zone of inhibition of 13 mm. The localized inhibition showed that the active phytoconstituents isolated in Fraction F5 were effective in reducing the growth of *Trichophyton rubrum*.

The results demonstrated that the antifungal chemicals were concentrated in fractions F4 and F5 by chromatographic separation. These results are in agreement with the TLC profile and justify the selection of these fractions for further phytochemical characterization by GC–MS and evaluation of their antifungal activity.

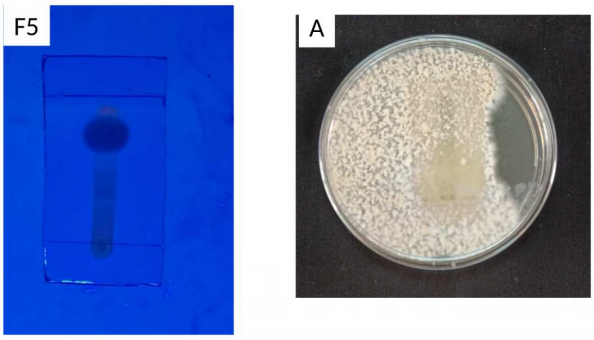


Figure 5. F5- TLC plates of ethanolic extract of *Piper betle* visualized under UV at 254 nm and 366 nm. A- TLC agar overlay bioautography of ethanolic extract of *Piper betle* against *Trichophyton rubrum*.

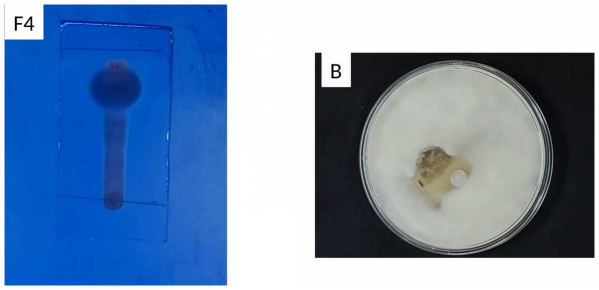
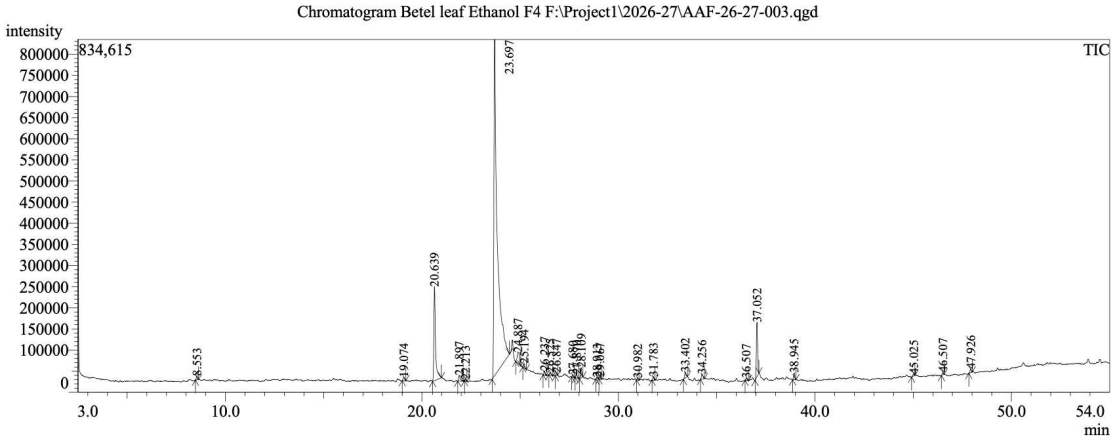


Figure 6. F4- TLC plates of ethanolic extract of *Piper betle* visualized under UV at 254 nm and 366 nm. B- TLC agar overlay bioautography of ethanolic extract of *Piper betle* against *Trichophyton mentagrophytes*.

Analysis of Bioactive Fractions F4 and F5 by Gas Chromatography–Mass Spectrometry (GC–MS)

GC-MS analysis was used to identify the phytochemical components of the bioactive chromatographic fractions (F4 and F5) derived from the ethanolic extract of *Piper betle*. Fraction F4 contained a total of 26 chemicals, while Fraction F5 contained 29 compounds. Phenolic compounds, terpenoids, sesquiterpenes, fatty acid derivatives, alcohols, and aromatic esters made up the majority of the discovered constituents. Many of these compounds have been shown to have antibacterial, antifungal, antioxidant, and anti-inflammatory qualities. Hydroxychavicol (76.73%) was the most prevalent compound found in Fraction F4, followed by Eugenol (10.36%), Phytol (4.41%), Benzoic acid, 4-ethoxy-, ethyl ester (1.64%), αR-Turmerone (0.77%), Methyleugenol (0.66%), 7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione (0.53%), Dibutyl phthalate (0.50%), and Caryophyllene oxide (0.32%). Lower amounts of minor ingredients were also found, including α-Cadinol, Neophytadiene, 1-Hexadecanol, Curlone, Tau-cadinol acetate, Tetracosane, Octacosane, and Tetratetracontane.Phenol, 2-methoxy-3-(2-propenyl)- (Eugenol) (8.22%), Phytol (4.13%), Benzoic acid, 4-ethoxy-, ethyl ester (1.46%), αR-Turmerone (0.87%), Dibutyl phthalate (0.81%), Ethyl oleate (0.69%), 2-Hexadecen-1-ol, 3,15-tetramethyl-, acetate (0.68%), Hexadecanoic acid, ethyl ester (0.38%), and Caryophyllene oxide (0.29%). Safrole, Humulene, Methyleugenol, Neophytadiene, Isopropyl myristate, Behenic alcohol, docosatrienoic acid methyl ester, Hexanedioic acid dioctyl ester, and heptadecane were among the other substances found in lesser amounts [28].These fractions are rich in physiologically active phytochemicals, as evidenced by the predominance of hydroxychavicol, eugenol, phytol, caryophyllene oxide, humulene, and other phenolic components. While phytol and caryophyllene oxide have been linked to antimicrobial, antioxidant, and cytoprotective qualities, hydroxychavicol and eugenol are well known for their broad-spectrum antimicrobial, antifungal, antioxidant, and anti-inflammatory activity. The strong antifungal activity seen against *Trichophyton mentagrophytes* and *Trichophyton rubrum* during Agar-based overlay technique bioautography and in vitro antifungal experiments may be explained by the enrichment of these chemicals in Fractions F4 and F5.

Table 8. Phytochemical constituents identified in Fraction F4 ethanolic extract of the *Piper betle* leaf by gas chromatography–mass spectrometry (GC–MS).



Peak#	R.Time	I.Time	F.Time	Area	Area%	Height%	Name	Similarity
1	8.553	8.480	8.625	36285	0.29	0.70	3,3-Diethoxybutan-2-one	93
2	19.074	19.000	19.180	40386	0.32	0.62	4-Terpinenyl acetate	86
3	20.639	20.540	20.990	1318134	10.36	16.23	Eugenol	97
4	21.897	21.820	22.115	84393	0.66	1.01	Methyleugenol	90
5	22.213	22.170	22.285	10377	0.08	0.23	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-	79
6	23.697	23.575	24.455	9760946	76.73	58.04	Hydroxychavicol	94
7	24.887	24.770	25.030	208786	1.64	1.95	Benzoic acid, 4-ethoxy-, ethyl ester	93
8	25.194	25.135	25.310	42776	0.34	0.73	2-Butenedioic acid (Z)-, dibutyl ester	88
9	26.237	26.170	26.330	40872	0.32	0.72	Caryophyllene oxide	86
10	26.525	26.470	26.665	31808	0.25	0.40	Diethyl Phthalate	83

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height%	Name	Similarity
11	26.847	26.790	26.915	22739	0.18	0.49	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-di	90
12	27.680	27.625	27.755	20920	0.16	0.43	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylet	88
13	27.870	27.800	27.995	43161	0.34	0.66	.alpha.-Cadinol	87
14	28.109	28.025	28.200	98361	0.77	1.77	aR-Turmerone	93
15	28.913	28.855	28.975	14626	0.11	0.30	Curlone	83
16	29.067	29.010	29.145	21604	0.17	0.44	3-Ethyl-2,6,10-trimethylundecane	83
17	30.982	30.920	31.050	20144	0.16	0.40	Tau-Cadinol acetate	81
18	31.783	31.715	31.865	35999	0.28	0.64	Neophytadiene	90
19	33.402	33.315	33.515	68029	0.53	1.06	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	85
20	34.256	34.180	34.375	63595	0.50	0.87	Dibutyl phthalate	92
21	36.507	36.445	36.615	24247	0.19	0.39	1-Hexadecanol	88
22	37.052	36.960	37.150	561458	4.41	9.37	Phytol	95

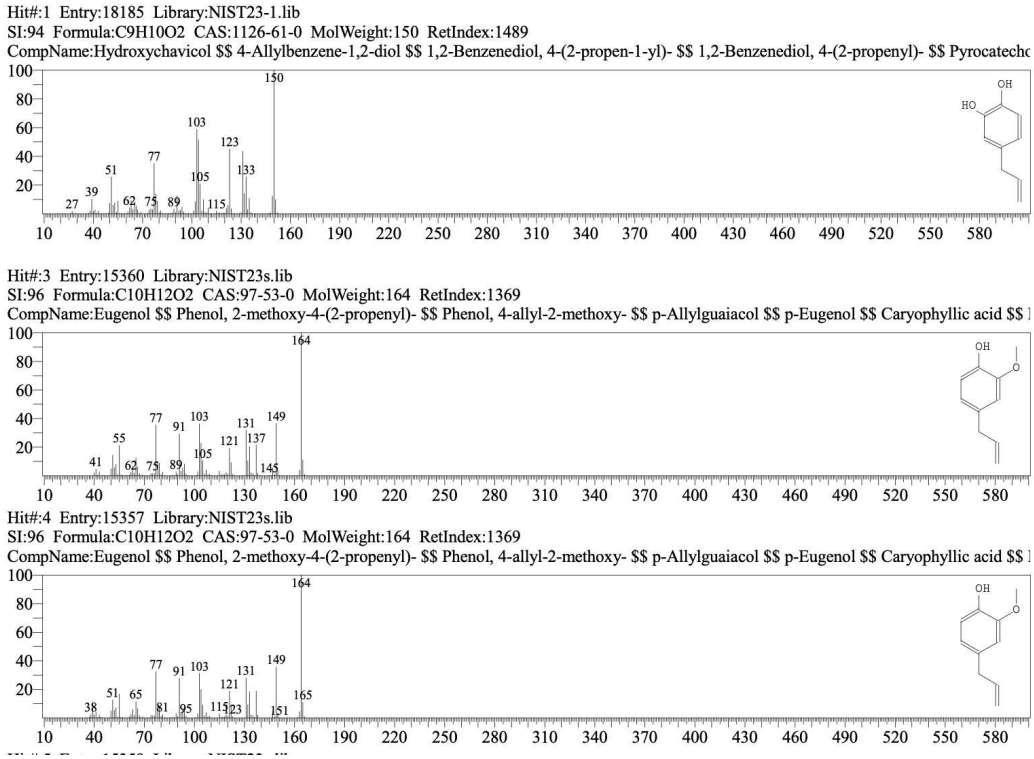
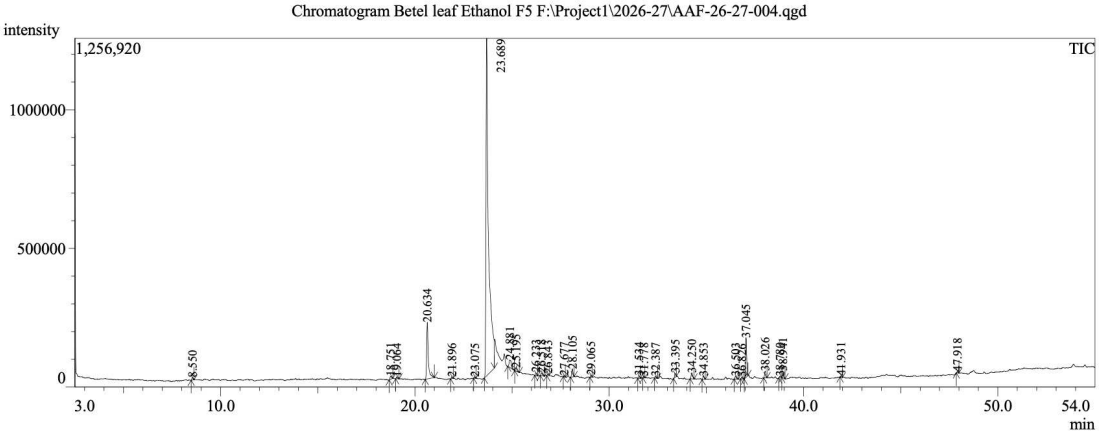


Table 9. Phytochemical constituents identified in Fraction F5 ethanolic extract of the *Piper betle* leaf by gas chromatography–mass spectrometry (GC–MS).



Peak#	R.Time	I.Time	F.Time	Area	Area%	Height%	Name	Similarity
1	8.550	8.485	8.630	37147	0.25	0.51	3,3-Diethoxybutan-2-one	89

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height%	Name	Similarity
2	18.751	18.670	18.865	58387	0.40	0.58	Safrole	91
3	19.064	19.005	19.180	31040	0.21	0.36	4-Terpinenyl acetate	87
4	20.634	20.530	20.995	1204226	8.22	10.96	Phenol, 2-methoxy-3-(2-propenyl)-	97
5	21.896	21.835	21.970	27559	0.19	0.38	Methyleugenol	90
6	23.075	23.020	23.140	19764	0.13	0.30	Humulene	88
7	23.689	23.570	24.085	11450383	78.16	65.25	Hydroxychavicol	94
8	24.881	24.775	25.115	214017	1.46	1.49	Benzoic acid, 4-ethoxy-, ethyl ester	95
9	25.195	25.130	25.365	63142	0.43	0.66	2-Butenedioic acid (Z)-, dibutyl ester	88
10	26.233	26.170	26.320	42806	0.29	0.58	Caryophyllene oxide	82
11	26.518	26.455	26.665	53844	0.37	0.53	Diethyl Phthalate	91
12	26.843	26.790	26.915	22026	0.15	0.33	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-di	89
13	27.677	27.635	27.745	14370	0.10	0.25	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylet	86
14	28.105	28.015	28.195	127805	0.87	1.56	aR-Turmerone	93
15	29.065	29.005	29.130	27745	0.19	0.42	Dodecane, 4,6-dimethyl-	85
16	31.534	31.470	31.615	36458	0.25	0.51	Isopropyl myristate	90
17	31.778	31.730	31.840	16228	0.11	0.27	Neophytadiene	89
18	32.387	32.335	32.500	25168	0.17	0.35	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	90
19	33.395	33.315	33.460	59219	0.40	0.85	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	89
20	34.250	34.165	34.370	118717	0.81	1.17	Dibutyl phthalate	95
21	34.853	34.780	34.945	55585	0.38	0.68	Hexadecanoic acid, ethyl ester	91
22	36.503	36.435	36.630	51506	0.35	0.56	Behenic alcohol	92

Piper betle ethanolic extract's cytotoxicity was assessed using the MTT assay on HaCaT cells.

After 48 hours of treatment, the MTT test was used to assess the cytotoxic potential of the ethanolic extract of *Piper betle* on HaCaT (human keratinocyte) cells [29]. At all tested concentrations (31.25–500 µg/mL), the extract showed a concentration-dependent decrease in cell viability. At concentrations of 31.25, 62.5, 125, 250, and 500 µg/mL, the percentage cell viability of HaCaT cells after treatment with the *Piper betle* extract was 91.21%, 51.84%, 25.74%, 13.78%, and 6.83%, respectively. A dose-dependent cytotoxic response was seen as the percentage inhibition rose from 8.79% at 31.25 µg/mL to 93.17% at 500 µg/mL. The assay's validity was confirmed by the positive control (doxorubicin, 1 µM), which showed 96.73% inhibition while untreated cells retained 100% viability. The ethanolic extract of *Piper betle* was shown to have a half-maximal inhibitory concentration (IC_{50}) of 60.58 µg/mL against HaCaT cells. Further morphological analysis showed that cellular damage increased with extract concentration, as seen by decreased cell density, shrinkage, loss of normal morphology, and separation from the culture surface. Only a tiny number of viable cells remained at the maximum concentration (500 µg/mL), indicating significant cytotoxicity [30,31].

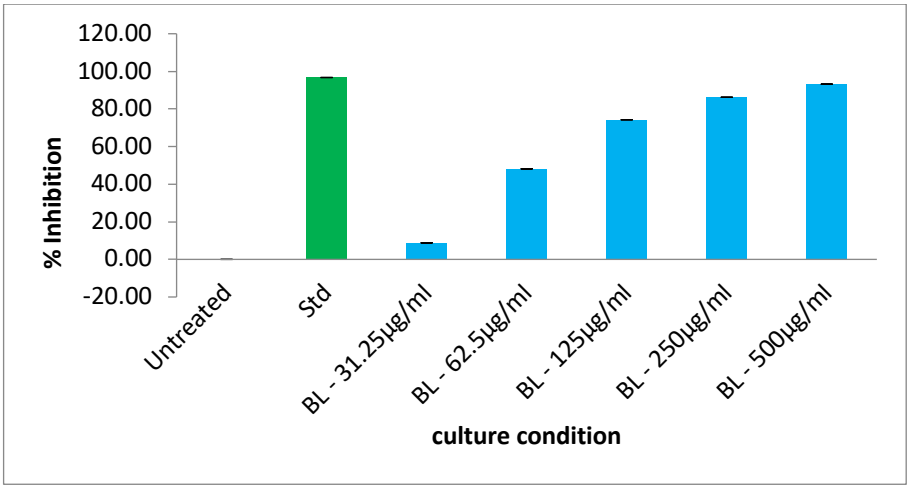
Overall, HaCaT cells showed notable concentration-dependent cytotoxic effects from the ethanolic extract of *Piper betle*. While dosages of 125–500 µg/mL caused noticeable decreases in cell viability, the comparatively high cell viability at 31.25 µg/mL implies decreased cytotoxicity at lower concentrations. These results demonstrate the extract's potent biological action and offer crucial information for choosing a safe concentration range for further antifungal activity.

Table 5. Details of drug treatment to respective cell line used for the study

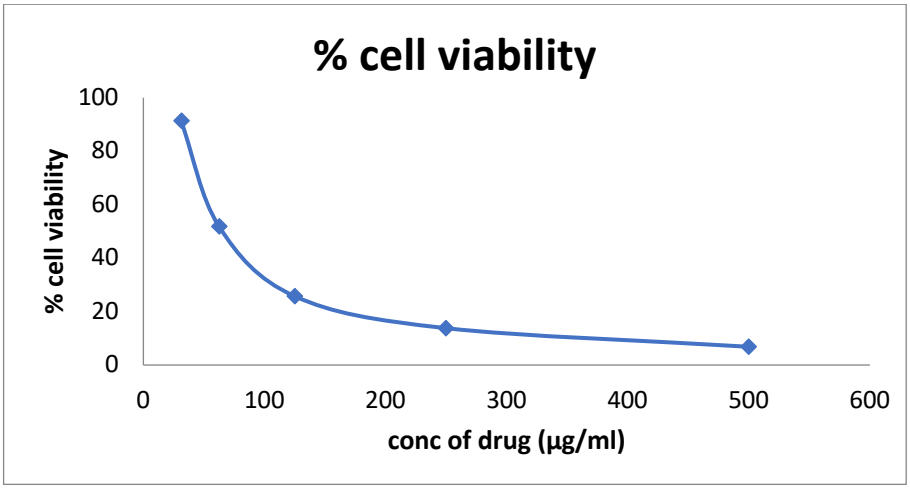
Sl.No	Culture condition	Cell lines	Concentration treated to cells
1	Untreated	HaCaT	No treatment
2	Blank	-	Only Media without cells
3	Std control (Dox)	HaCaT	1 (1uM/ml)
4	*Piper betle* extract	HaCaT	31.25µg/ml
5	*Piper betle* extract	HaCaT	62.5µg/ml
6	*Piper betle* extract	HaCaT	125µg/ml
7	*Piper betle* extract	HaCaT	250µg/ml
8	*Piper betle* extract	HaCaT	500µg/ml

Table 6. % cell viability values of sample BL along with controls against HaCaT cells after the treatment period of 48 hrs.

Sample code	Concentration	Cells	% cell viability ± SE	% cell inhibition ± SE
Untreated	No treatment	HaCaT	100 ± 0.002	0.00 ± 0.002
Dox-1uM	0.531 µg/mL	HaCaT	3.27 ± 0.001	96.73 ± 0.001
Piper betle extract	31.25µg/ml	HaCaT	91.21 ± 0.001	8.79 ± 0.001
Piper betle extract	62.5µg/ml	HaCaT	51.84 ± 0.002	48.16 ± 0.002
Piper betle extract	125µg/ml	HaCaT	25.74 ± 0.0015	74.26 ± 0.0015
Piper betle extract	250µg/ml	HaCaT	13.78 ± 0.0015	86.22 ± 0.0015
Piper betle extract	500µg/ml	HaCaT	6.83 ± 0.0005	93.17 ± 0.0005



Graph 1. % cell inhibition values of sample BL against HaCaT cells after the incubation period of 48hrs along with controls.



Graph 2. % cell viability of HaCaT cells after treatment with Piper betle leaf extract (31.25–500 µg/ml) for 48 hrs.

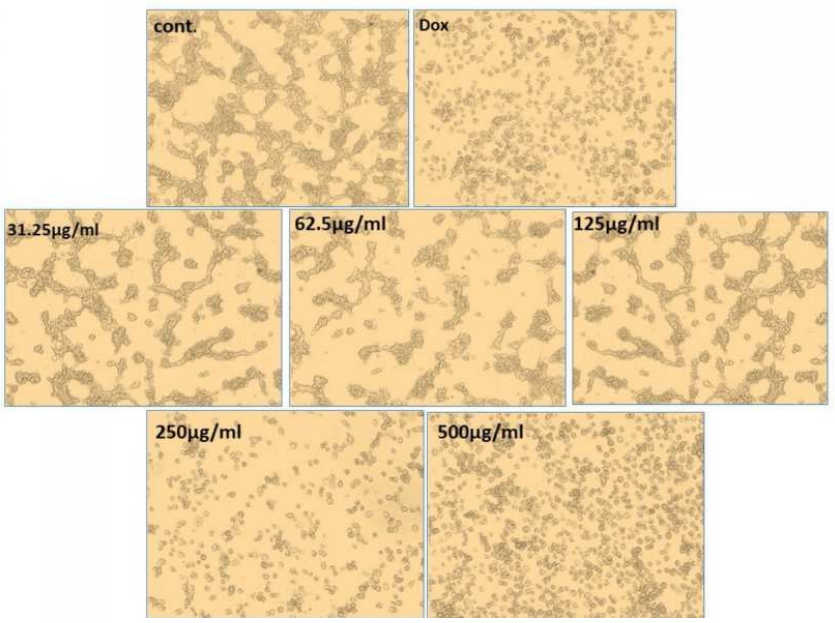


Figure 7. % cell inhibition images of sample *Piper betle* leaf extract against HaCaT cells after the incubation period of 48hrs along with controls.

Discussion

The findings of the present study demonstrate that selected medicinal plant extracts possess measurable antifungal activity against two clinically significant dermatophytes, *T. rubrum* and *T. mentagrophytes*. Among the 14 plants initially screened, *Piper betle* emerged as the most potent, with its ethanolic extract producing nearly complete inhibition of mycelial growth (99.1% against *T. rubrum* and 95.2% against *T. mentagrophytes* at 20 mg/mL). These inhibition values are particularly noteworthy because they exceeded those observed for the standard antifungal drug ketoconazole at 25 µg/mL, which showed 76.5% and 74.5% inhibition against *T. rubrum* and *T. mentagrophytes*, respectively.

The strong antifungal performance of *Piper betle* can be attributed to its rich phytochemical profile. The ethanol extract of this plant was found to contain alkaloids, tannins, flavonoids, phenols, glycosides, and terpenoids, all of which have been independently associated with antifungal activity in the literature. Phenolic compounds, in particular, are known to disrupt fungal cell membranes and interfere with mitochondrial function, while flavonoids can inhibit spore germination and hyphal growth [6,7]. The synergistic interaction among these bioactive constituents may explain the extract's superior performance compared with the individual plant extracts that contained fewer classes of secondary metabolites.

The MIC results further support the antifungal potential of these plant extracts. The lowest MIC value recorded was 0.312 mg/mL, observed for *Curcuma longa* ethanol extract against T. rubrum and for both *Piper longum* and *Mangifera indica* against *T. mentagrophytes*. These values compare favourably with those reported in earlier studies. Espinel-Ingroff et al. [14] established reference MIC ranges for synthetic antifungals such as voriconazole and amphotericin B against filamentous fungi, and the MIC values obtained in our study for the plant extracts fall within a range that suggests practical therapeutic relevance. The observation that different extracts showed varying MIC values against the two *Trichophyton* species indicates that the susceptibility of dermatophytes to plant-derived compounds is species-dependent, a finding that is consistent with the differential antifungal susceptibility patterns reported for synthetic drugs [15].

The remaining selected plants—*Trigonella foenum-graecum*, *Mangifera indica*, and *Curcuma longa*—also exhibited considerable antifungal activity, with percentage inhibition values ranging from approximately 60% to 66% against *T. mentagrophytes*. Their phytochemical profiles, which included combinations of alkaloids, terpenoids, phenols, and glycosides, are consistent with the classes of compounds that have been shown to disrupt fungal cell wall integrity and inhibit essential metabolic pathways in fungi [6]. Notably, the extracts of *Senegalia ruguata*and *Nerium oleander* showed the weakest activity, with inhibition values below 28%, suggesting that these species may not be suitable candidates for further antifungal drug development, at least not in the solvent systems employed in this study.

Our findings are broadly consistent with previous reports in the literature. Savarirajan et al. [16] demonstrated that *Cassia occidentalis* seed extract and Asparagus racemosus leaf extract exhibited antifungal activity against *Microsporum gypseum*, *M. canis*, *T. mentagrophytes*, and *T. terrestre*. Similarly, Balakumar et al. [17] reported antifungal activity of *Ocimum sanctum* extracts against clinically isolated dermatophytes. Barros Cota et al. [18] described antifungal effects of saponin-rich extracts from *Chamaecostus* cuspidatus against *Candida* and *Trichophyton* species. What distinguishes the present investigation, however, is the

remarkably high inhibition exhibited by *Piper betle*, which, to our knowledge, has not been previously reported at these magnitudes against T. rubrum and *T. mentagrophytes*. In order to separate the antifungal components of *Piper betle* leaf extract against *Trichophyton rubrum* and *Trichophyton mentagrophytes*, the current work used bioassay-guided fractionation. The presence of alkaloids, flavonoids, phenolic compounds, tannins, saponins, glycosides, and terpenoids was confirmed by preliminary phytochemical screening, suggesting that *Piper betle* varied phytochemical makeup is linked to its antifungal effect. Medicinal plants with broad-spectrum antibacterial activity have been found to have similar phytochemical profiles, with phenolics and flavonoids playing a major role in their biological characteristics.

Using a chloroform acetate (7:3, v/v) solvent solution, bioassay-guided column chromatography effectively separated the crude ethanolic extract into six fractions. F4 and F5 showed the greatest antifungal activity among the collected fractions, suggesting that the active ingredients were concentrated in these fractions. After chromatographic separation, the antifungal activity gradually increased, indicating that purification increased the concentration of bioactive chemicals by eliminating inactive components from the crude extract. Similar findings have been documented in research where the enrichment of active phytochemicals through chromatographic fractionation increased the biological activity of extracts from medicinal plants.

The successful separation of chemically unique molecules was further confirmed by thin-layer chromatography, which showed that F4 and F5 contained well-resolved phytochemical elements with distinct Rf values. The antifungal chemicals were targeted directly on the TLC plate using Agar-based overlay technique bioautography, demonstrating a direct correlation between chromatographic separation and biological activity. While fraction F5 showed the best inhibitory efficacy against Trichophyton rubrum, fraction F4 formed a 16 mm inhibition zone against Trichophyton mentagrophytes. These results reinforce the usefulness of bioassay-guided fractionation for identifying active elements by showing that the antifungal activity is linked to particular chromatographic bands rather than the full crude extract.

Hydroxychavicol was found to be the most prevalent compound in the bioactive fractions, followed by eugenol, phytol, caryophyllene oxide, αR-turmerone, and numerous other phenolic and terpenoid compounds, according to GC-MS analysis. Previously identified as the main bioactive phenolic ingredient in *Piper betle*, hydroxychavicol demonstrates broad-spectrum antibacterial and antifungal properties through oxidative stress generation, enhanced membrane permeability, and loss of fungal membrane integrity. Eugenol's capacity to prevent fungal growth via modifying membrane function and inhibiting ergosterol biosynthesis is also well-established. Similarly, the antibacterial, antioxidant, and anti-inflammatory properties of phytol and caryophyllene oxide may enhance the overall antifungal effect.

The enrichment of these bioactive phenolic chemicals during chromatographic separation is probably responsible for the increased antifungal activity seen in fractions F4 and F5. The observed antifungal effect is probably the consequence of synergistic interactions between hydroxychavicol, eugenol, and other minor phytochemicals discovered by GC-MS rather than a single component working alone. Numerous medicinal plants have been shown to display these synergistic interactions, with phenolic compound combinations showing more biological activity than individual elements.

Overall, a methodical approach to determining *Piper betle* antifungal principles was made possible by the combined use of column chromatography, TLC, Agar-based overlay technique bioautography, and GC–MS [26,27]. The antifungal potential of *Piper betle* is mainly linked to phenolic and terpenoid elements concentrated in fractions F4 and F5, as confirmed by the agreement between chromatographic separation, localization of antifungal activity, and chemical analysis. These results show *Piper betle* potential as a source of natural antifungal agents for the creation of topical treatments against dermatophytosis and offer compelling scientific evidence in favour of its traditional use in the treatment of fungal diseases.

Using the poisoned food technique and broth microdilution minimum inhibitory concentration (MIC) assay, the present study assessed the antifungal activity of five medicinal plant extracts against Trichophyton rubrum and Trichophyton mentagrophytes: Trigonella foenum-graecum, Curcuma longa, Piper betle, Mangifera indica, and Piper longum. Although the degree of inhibition varied across the plants, all five extracts showed inhibitory action against the tested dermatophytes. These discrepancies could be explained by variations in the concentration of bioactive components, extraction efficiency, and phytochemical makeup.

Piper betle showed the most potent antifungal activity of all the studied extracts. This finding is in line with earlier studies showing that P. betel has broad-spectrum antifungal capabilities against dermatophytes. According to Ali et al. (2010), the primary phenolic component of P. betel, hydroxychavicol, demonstrates strong fungicidal action by compromising the integrity of fungal cell membranes, increasing membrane permeability, and causing intracellular components to seep out. Therefore, the concentration of phenolic chemicals, essential oils, and flavonoids in the extract is probably connected to the significant antifungal activity seen in this study. Weitzman and Summerbell (1995) have found similar findings, emphasizing the effectiveness of phenolic-rich medicinal herbs as dermatophyte growth inhibitors.

Out of all the plants studied, Curcuma longa showed the second-highest antifungal activity. Curcuminoids, especially curcumin, which disrupt fungal cell membrane production, prevent ergosterol biosynthesis, cause oxidative stress, and compromise mitochondrial function, have been generally linked to turmeric's antifungal properties. Curcumin has the potential to be a natural antifungal drug because prior research has demonstrated its inhibitory efficacy against a number of pathogenic fungus, including dermatophytes. Therefore, the current investigation's results are consistent with existing publications that show C. longa extracts can inhibit fungal growth through a variety of complementary methods.

Significant antifungal activity was also demonstrated by the Trigonella foenum-graecum methanolic extract. Steroidal saponins, diosgenin, flavonoids, tannins, alkaloids, and phenolic acids have all been linked to antibacterial qualities in fenugreek seeds. These substances are thought to impede fungal metabolism, interfere with enzyme activity, and enhance fungal membrane permeability. Although T. foenum-graecum's antifungal activity is often lower than Piper betle's, other studies have demonstrated its efficacy against dermatophytes and other pathogenic fungi, corroborating the results of this work.

The leaf extract of Mangifera indica showed significant antifungal efficacy against both kinds of dermatophytes. Mangiferin, gallic acid, quercetin, catechins, and other polyphenolic chemicals have been identified as the main sources of M. indica's antifungal qualities. These phytochemicals work by inducing oxidative stress, disrupting membranes, and inhibiting fungal enzyme systems. The antifungal action demonstrated in this study is consistent with previous research showing that M. indica extracts suppress the growth of Trichophyton species.

Additionally, Piper longum showed encouraging antifungal action. Piperine, piperlongumine, lignans, and volatile oils—all of which have antifungal, anti-inflammatory, and antioxidant qualities—have been linked to P. longum's biological activity. By changing cellular metabolism and membrane permeability, piperine has been shown to disrupt fungal membrane function and prevent fungal growth. Thus, the current study's moderate to high antifungal activity is in line with other findings emphasizing P. longum's therapeutic potential against fungal infections.

While the broth microdilution MIC assay measured the lowest dosage necessary to inhibit fungal growth, the poisoned food approach showed distinct variations in radial mycelial growth inhibition across the investigated plant extracts. The poisoned food test assesses inhibition of hyphal extension on solid media, whereas the MIC assay analyses suppression of fungal growth in liquid culture, which explains the minor discrepancies between % inhibition and MIC values. The numerical values obtained in each assay may therefore be affected by variations in compound diffusion through agar, solubility in broth, molecular size, and chemical stability. However, Piper betle and Curcuma longa were consistently placed among the most active extracts by both methods, indicating that the antifungal activity was repeatable [37,38].

Overall, the current study's results are consistent with other reports detailing the antifungal efficacy of medicinal herbs against dermatophytes. The greater activity of Curcuma longa and Piper betle, followed by Trigonella foenum-graecum, Mangifera indica, and Piper longum, indicates that these plants have strong bioactive chemicals that can stop Trichophyton species from growing. These findings lend credence to their continued phytochemical analysis, active component extraction, and future development as natural antifungal agents for the treatment of dermatophytic infections. The broth microdilution MIC assay and the poisoned food technique measure distinct characteristics of antifungal activity and may thus not give identical scores of plant extracts. The poisoned food technique consists of incubation on solid agar for lengthy periods and assesses the inhibition of radial mycelial growth, which is an indicator of the ability of phytochemicals to limit hyphal elongation. Conversely, the broth microdilution assay measures the minimum concentration required to prevent the visible development of fungus in a liquid media under standardized conditions. Differences in compound solubility, polarity, diffusion, stability, protein binding and interaction with agar or broth can impact the availability of phytochemicals and hence their apparent antifungal efficacy. The present investigation on Piper betle exhibited the highest inhibition of mycelial development in the poisoned food assay which indicated excellent suppression of hyphal extension on solid medium. However, Curcuma longa displayed a lower MIC against T. rubrum, indicating a higher inhibitory efficacy under broth microdilution conditions. These findings are not inconsistent but reflect the distinct biological objectives and the varied physicochemical settings of the two experiments. Therefore, both approaches can be regarded as complimentary tools for the evaluation of the antifungal potential of crude plant extracts.

Conclusion

This study provides evidence that selected medicinal plant extracts, particularly the ethanolic extract of Piper betle, possess significant antifungal activity against the dermatophytes *T. rubrum* and *T. mentagrophytes* under in vitro conditions. Among the 14 plants screened, five—*Trigonella foenum-graecum*, *Piper betle*, *Curcuma longa*, *Piper longum*, and *Mangifera indica*—were identified as the most active, with *Piper betle* demonstrating the highest percentage inhibition of mycelial growth. The MIC values obtained for *Curcuma longa*, *Piper longum*, and *Mangifera indica* (0.312 mg/mL) suggest that these extracts contain potent antifungal constituents at relatively low concentrations.

The broth microdilution MIC assay and the poisoned food technique measure different characteristics of antifungal activity and may not provide identical scores of plant extracts. The poisoned food technique consists of incubation on solid agar for long periods and measures the inhibition of radial mycelial growth, which is an indicator of the capacity of phytochemicals to inhibit hyphal elongation. The broth microdilution assay, however, measures the minimum concentration of an agent needed to prevent visible development of fungus in a liquid media under standardized conditions. Differences in compound solubility, polarity, diffusion, stability, protein binding and interaction with agar or broth can affect the availability of phytochemicals and thus their apparent antifungal efficacy. In the poisoned food assay, the present investigation on Piper betle showed the highest inhibition of mycelial development, which means excellent suppression of hyphal extension on solid medium. However, Curcuma longa showed lower minimum inhibitory concentration (MIC) against T. rubrum indicating higher inhibitory efficacy under broth microdilution conditions. These findings are not contradictory but reflect the different biological objectives and the different physicochemical settings of the two experiments. Thus, both methods can be regarded as complementary tools to assess the antifungal potential of crude plant extracts.

Phytochemical analysis confirmed the presence of several classes of secondary metabolites in the active extracts, including alkaloids, tannins, flavonoids, phenols, saponins, glycosides, and terpenoids, which collectively may be responsible for the observed antifungal effects. These findings lend scientific support to the traditional use of these plants in managing fungal skin infections and highlight their potential as sources of natural antifungal agents.

The selected formulations showed higher antifungal activity than the individual plant extracts among the herbal combinations studied, indicating a synergistic impact of the phytochemicals present. Bioassay-guided fractionation by column chromatography and TLC led to the separation of bioactive fractions, with Fractions F4 and F5 showing the highest antifungal activity [25,26]. The active antifungal ingredients were further validated by the Agar-based overlay technique bioautography where the fraction F4 showed zone of inhibition of 16 mm against *Trichophyton mentagrophytes* and fraction F5 showed strong inhibitory activity against *Trichophyton rubrum*.

GC–MS examination of the active fractions showed the preponderance of hydroxychavicol, eugenol, phytol, αR-turmerone, caryophyllene oxide and a number of other phenolic and terpenoid chemicals[27]. These phytoconstituents have been widely reported for their antifungal, antimicrobial, antioxidant and antiinflammatory characteristics which might be responsible for the observed biological activity.

Scanning electron microscopy (SEM) further showed that treatment with the bioactive fractions caused severe ultrastructural damage in both dermatophytes. Treated fungal hyphae showed rough surfaces, wrinkled cell walls, collapse of normal tubular hyphae, distorted branching, fragmentation of the mycelial network, shrinkage, rupture and deformation of fungal structures compared with the untreated controls indicating disruption of cell wall and membrane integrity resulting in irreversible cellular damage.

The cytotoxicity evaluation on the HaCaT human keratinocyte cells showed a concentration dependent decrease in cell viability with an IC_{50} value of 60.58 µg/mL, providing vital information on the biological activity and safe concentration range of the extract for topical applications [31,32,33].

Overall, the results of the present investigation give scientific proof that the Piper betle leaf extract is a viable natural source of antifungal chemicals for the management of dermatophytosis. The therapeutic potential of the active fractions is validated using the combination of phytochemical profiling, bioassay-guided fractionation, GC–MS characterization, Agar-based overlay technique bioautography, SEM analysis, and cytotoxicity evaluation.

References

1. Lakshmipathy, D.T. and Kannabiran, K., 2010. Dermatomycosis: pathogenesis and treatment. Natural Science, 2(7), pp.726–731.
2. Ali-Shtayeh, M.S. and Arda, H.M., 1986. A study of tineasis in Jordan (West Bank). Journal of Tropical Medicine and Hygiene, 89, pp.137–141.
3. Branda, F., Petrosillo, N., Ceccarelli, G., Giovanetti, M., De Vito, A., Madeddu, G., Scarpa, F. and Ciccozzi, M., 2025. Antifungal agents in the 21st century: advances, challenges, and future perspectives. Infectious Disease Reports, 17(4), p.91.
4. CDC, 2019. Antibiotic Resistance Threats in the United States, 2019. U.S. Department of Health and Human Services, CDC, Atlanta, GA.
5. WHO, 2013. WHO Traditional Medicine Strategy: 2014–2023. World Health Organization, Geneva.
6. Cowan, M.M., 1999. Plant products as antimicrobial agents. Clinical Microbiology Reviews, 12(4), pp.564–582.
7. Gruz, J., Novak, O., Strnad, M. and Teixeira da Silva, J.A., 2011. Cycloaurone-type flavonoids in the genus Cyclopia and their biological activities. Phytochemistry, 72(8), pp.945–955.
8. Abirami, S., Raj, B.E., Soundarya, T., Kannan, M., Sugapriya, D., Al-Dayan, N. and Mohammed, A.A., 2021. Exploring antifungal activities of acetone extract of selected Indian medicinal plants against human dermal fungal pathogens. Saudi Journal of Biological Sciences, 28(4), pp.2180–2187.
9. Kedia, A., Prakash, B., Mishra, P.K., Chanotiya, C.S. and Dubey, N.K., 2014. Antifungal, antiaflatoxigenic, and insecticidal efficacy of spearmint (Mentha spicata L.) essential oil. International Biodeterioration & Biodegradation, 89, pp.29–36.
10. Prakash, B., Mishra, P.K., Kedia, A. and Dubey, N.K., 2014. Antifungal, antiaflatoxin and antioxidant potential

of chemically characterized Boswellia carterii Birdw essential oil and its in vivo practical applicability in preservation of Piper nigrum L. fruits. LWT-Food Science and Technology, 56(2), pp.240–247.

11. Smith, H., Doyle, S. and Murphy, R., 2015. Filamentous fungi as a source of natural antioxidants. Food Chemistry, 185, pp.389–397.
12. CLSI, 2008. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard, 2nd ed. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
13. Espinel-Ingroff, A., Bartlett, M., Bowden, R., Chin, N.X., Cooper Jr, C., Fothergill, A., McGinnis, M.R., Menezes, P., Messer, S.A., Nelson, P.W. and Odds, F.C., 1997. Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous fungi. Journal of Clinical Microbiology, 35(1), pp.139–143.
14. Espinel-Ingroff, A., Cuenca-Estrella, M., Fothergill, A., Fuller, J., Ghannoum, M., Johnson, E., Pelaez, T., Pfaller, M.A. and Turnidge, J., 2011. Wild-type MIC distributions and epidemiological cutoff values for amphotericin B and Aspergillus spp. for the CLSI broth microdilution method (M38-A2 document). Antimicrobial Agents and Chemotherapy, 55(11), pp.5150–5154.
15. Debourgogne, A., De Hoog, S., Lozniewski, A. and Machouart, M., 2012. Amphotericin B and voriconazole susceptibility profiles for the Fusarium solani species complex: comparison between the E-test and CLSI M38-A2 microdilution methodology. European Journal of Clinical Microbiology & Infectious Diseases, 31(4), pp.615–618.
16. Savarirajan, D., et al., 2021. Antifungal activity of Cassia occidentalis seed extract and Asparagus racemosus leaf extract against dermatophytes. Journal of Pharmacognosy and Phytochemistry, 10(2), pp.132–139.
17. Balakumar, S., Rajan, S., Thirunalasundari, T. and Jeeva, S., 2011. Antifungal activity of Ocimum sanctum Linn. (Lamiaceae) on clinically isolated dermatophytic fungi. Asian Pacific Journal of Tropical Medicine, 4(8), pp.654–657.
18. Barros Cota, B., Batista Carneiro de Oliveira, D., Carla Borges, T., Cristina Catto, A., Valverde Serafim, C., Rogelis Aquiles Rodrigues, A., Kohlhoff, M., Leomar Zani, C. and Assuncao Andrade, A., 2021. Antifungal activity of extracts and purified saponins from the rhizomes of Chamaecostus cuspidatus against Candida and Trichophyton species. Journal of Applied Microbiology, 130(1), pp.61–75.
19. Ennaghra, N., Bourzama, G. and Soumati, B., 2019. Effect of aqueous extract of Eucalyptus sp., Nerium oleander and Allium sativum on the growth of dermatophytes. Fungal Territory, 2, pp.6–11.
20. Farjana, A., Zerin, N. and Kabir, M.S., 2014. Antimicrobial activity of medicinal plant leaf extracts against pathogenic bacteria. Asian Pacific Journal of Tropical Disease, 4, pp.S920–S923.
21. Ogidi, O.C. and Oyetayo, V.O., 2016. Phytochemical screening and assessment of antidermatophytic activity of some selected macrofungi against pathogenic dermatophytes. Mycobiology, 7(1), pp.9–14.
22. Rezgui, M., Majdoub, N., Mabrouk, B., Baldisserotto, A., Bino, A., Kaab, L.B. and Manfredini, S., 2020. Antioxidant and antifungal activities of marrubiin, extracts and essential oil from Marrubium vulgare L. against pathogenic dermatophyte strains. Journal de Mycologie Medicale, 30(1), p.100927.
23. Kang, F., Wei, R., Huang, X., Dawuda, U.K., et al., 2023. The effect of plant extracts on growth performance, immune response, and disease resistance in aquaculture. Aquaculture, 567, p.739634.
24. Espinel-Ingroff, A., Dawson, K., Pfaller, M., Anaissie, E., Breslin, B., Dixon, D., Fothergill, A., Paetznick, V., Peter, J. and Rinaldi, M., 1995. Comparative and collaborative evaluation of standardization of antifungal susceptibility testing for filamentous fungi. Antimicrobial Agents and Chemotherapy, 39(2), pp.314–319.
25. El-Bondkly, E.A.M., Al Shammari, B., El-Gendy, M.M.A.A., Alsafari, I.A., El-Bondkly, A.A.M.A., El-Shenawy, F.S. and El-Bondkly, A.M., 2022. Phytochemical screening, antifungal, and anticancer activities of medicinal plants Thymelaea hirsuta, Urginea maritima, and Plantago albicans. *BioMed Research International*, *2022*(1), p.9544915.
26. Valle Jr, D.L., Puzon, J.J.M., Cabrera, E.C. and Rivera, W.L., 2016. Thin layer chromatography-bioautography and gas chromatography-mass spectrometry of antimicrobial leaf extracts from Philippine Piper betle L. against multidrug-resistant bacteria. *Evidence-Based Complementary and Alternative Medicine*, *2016*(1), p.4976791.
27. Thamaraikani, I. and Kulandhaivel, M., 2017. Purification of hydroxychavicol from Piper betle Linn and evaluation of antimicrobial activity against some food poison causing bacteria. *Journal of Pure and Applied Microbiology*, *11*(4), pp.1883-1889.
28. Thanh, N.P.T., Pham, P.D., Hoang, D.M., Kim, M.T.N. and Tran, T.T., 2024. Extracting hydroxychavicol and evaluating the antibacterial and antifungal properties of betle leaf extract (Piper Betle L.). *Chemical Engineering Transactions*, *113*, pp.343-348.
29. MTT Cell Proliferation Assay Instruction Guide – ATCC, VA, USA
30. GerlierD., and N. ThoS2set. J. Immunol. Methods 94: 57-63, 1986. Alley, M.C.
31. Cancer Res. 48: 589-601, 1988. Mosmann, T. J. Immunol. Methods 65: 55-63, 1983.
32. Alley, M. C., Scudiere, D. A., Monks, A., Czerwinski, M., Shoemaker, R. II., and Boyd, M. R. Validation of an automated microculturetetrazolium assay (MTA) to assess growth and drug sensitivity of human tumor cell lines. Proc. Am. Assoc. Cancer Res., 27: 389, 1986
33. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983; 65: 55–63.
34. Weitzman, I., & Summerbell, R. C. (1995). *The dermatophytes*. Clinical Microbiology Reviews, 8(2), 240–259.
35. Ali, I., et al. (2010). In vitro antifungal activity of hydroxychavicol isolated from *Piper betle* L. Annals of Clinical Microbiology and Antimicrobials, 9, 7.
36. Cowan, M. M. (1999). Plant products as antimicrobial agents. Clinical Microbiology Reviews, 12(4), 564–582.
37. Mukherjee, P. K., et al. (2021). Development of natural products in drug discovery against fungal infections. Journal of Ethnopharmacology, 267, 113463.

37. Esteban, A., Abarca, M. L., & Cabañes, F. J. (2005). Comparison of disk diffusion method and broth microdilution method for antifungal susceptibility testing of dermatophytes. *Medical mycology*, *43*(1), 61-66.
38. Niewerth, M., Splanemann, V., Korting, H. C., Ring, J., & Abeck, D. (1998). Antimicrobial susceptibility testing of dermatophytes–comparison of the agar macrodilution and broth microdilution tests. *Chemotherapy*, *44*(1), 31-35.