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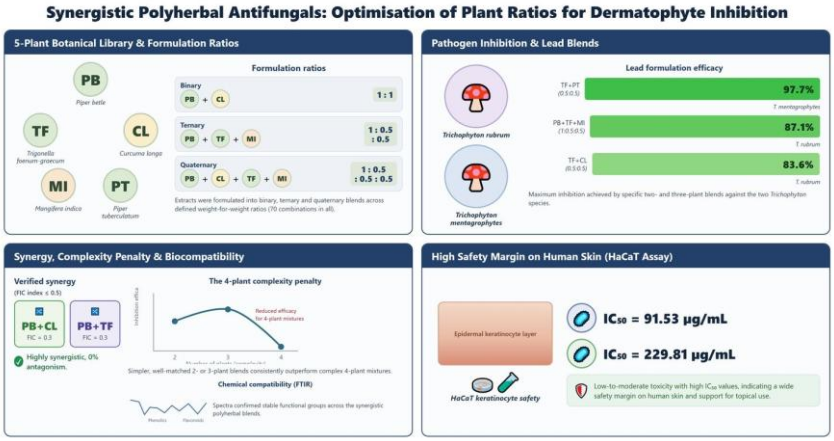
Synergistic Antifungal Activity of Polyherbal Combinations against Trichophyton rubrum and Trichophyton mentagrophytes: In Vitro Evaluation, FTIR Characterization and Cytotoxicity Assessment

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ABSTRACT: Dermatophytosis caused by Trichophyton rubrum and Trichophyton mentagrophytes remains one of the most common superficial fungal infections, and terbinafine- and azole-resistant strains are now reported often enough to worry clinicians [1,2]. Plant combinations offer a practical route to overcome this resistance. This study combined solvent extracts of five medicinal plants — Piper betle (PB), Trigonella foenum-graecum (TF), Curcuma longa (CL), Mangifera indica (MI) and Piper tuberculatum (PT) — into binary, ternary and quaternary blends and screened all 70 of them against the two pathogens by the poisoned-food technique. The ratio was as important as the pairing of plants. The strongest binary blend against T. rubrum was TF+CL (0.5:0.5), which reduced mycelial growth by 83.6 ± 1.5%; against T. mentagrophytes, it was TF+PT (0.5:0.5), reaching 97.7 ± 2.2%. The best three-plant blend, PB+TF+MI (1:0.5:0.5), lifted the inhibition of T. rubrum to 87.1 ± 0.5%, yet the four-plant mixtures were consistently weaker than the simpler ones. MIC and FIC-index testing of the most active blends flagged three synergistic pairings against T. rubrum and one against T. mentagrophytes; the rest were additive or indifferent, and none were antagonistic [3]. The FTIR spectra of the synergistic blends exhibited hydroxyl, aliphatic C–H, carbonyl, aromatic C=C, and C–O bands, which are expected in phenolics, flavonoids, terpenoids, and glycosides. On HaCaT keratinocytes, the two lead blends gave IC_{50} values of 91.53 µg/mL (PB+CL) and 229.81 µg/mL (PB+CL+TF+MI), so both remained reasonably safe at the doses that suppressed the fungi. The practical message is simple: two well-matched plants outperform an elaborate mixture, and they do so with little harm to the skin cells.

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Graphical abstract. The five-plant botanical library and formulation ratios demonstrated lead-blend antifungal efficacy against the two dermatophytes, verified synergy with the four-plant complexity penalty and FTIR compatibility, and ensured a safety margin for HaCaT keratinocytes.

1. INTRODUCTION

Approximately one in four people experience superficial fungal infections, most commonly affecting the skin, hair, or nails [1,4]. Dermatophytes are responsible for most of these cases. They are keratinophilic filamentous fungi of the genera *Trichophyton*, *Microsporum*, and *Epidermophyton*, among which *Trichophyton rubrum* and *Trichophyton mentagrophytes* are most often found in onychomycosis, tinea corporis, tinea cruris, and tinea pedis [4]. These fungi feed on the keratin of the outer skin, and the infections they cause tend to linger, recur, and spread, which places a steady load on dermatology clinics across both temperate and tropical regions.

Treatment has long relied on two drug classes. Azoles and allylamines are administered either as brief topical courses or as longer oral regimens, depending on the location and severity of the infection [5]. These agents are effective in most patients; however, prolonged use can lead to hepatotoxicity, awkward drug interactions, and treatment failure. Dermatophyte resistance is a major concern. Mutations in the squalene epoxidase and 14α-demethylase genes, together with overactive efflux pumps, allow many isolates to shrug off terbinafine and azoles [2,6]. This pattern echoes the broader crisis of antimicrobial resistance and has pushed researchers to search for alternatives [7].

Combination therapy is a promising treatment option. Pairing two agents that hit different fungal targets can produce synergy, widen the spectrum, and drop the dose each agent has to reach, which in turn eases the selective pressure that breeds resistance [3,8]. Medicinal plants are well suited to this logic. A single extract already holds a tangle of phenolics, flavonoids, terpenoids, alkaloids, and curcuminoids, which act through several antifungal routes at once — tearing at membranes, blocking ergosterol synthesis, tipping cells into oxidative stress, or jamming fungal enzymes [9,10,11]. Mixing extracts from different plants can reinforce one another, resulting in effects greater than those of any single extract [12,13,14]. However, few studies have systematically examined plant–plant interactions against dermatophytes, and even fewer have examined how the mixing ratio or the number of partners changes the outcome [8,15].

Each of the five plants chosen in this study has a documented case of antifungal use. *Piper betle* leaves are rich in hydroxychavicol and related phenolics that exhibit broad antimicrobial activity [16]. *Curcuma longa* rhizomes supply curcuminoids that both curb fungal growth and enhance the action of azoles [17,18,19]. *Trigonella foenum-graecum* seeds contain saponins and flavonoids with reported antifungal activity [20,21]. *Mangifera indica* leaves contain mangiferin and flavonoids that affect filamentous fungi [22,23], and *Piper tuberculatum* contains piperamides and terpenoids with recognized bioactivity [24]. Despite this rich pharmacology, the literature lacks a clear account of how these extracts behave when combined against dermatophytes and whether adding more plants actually helps.

This study aims to answer this question. Binary, ternary, and quaternary combinations of the five plants were screened in defined proportions against *T. rubrum* and *T. mentagrophytes*, giving 70 blends in all. The nature of each interaction was determined using MIC and FIC-index testing, the functional groups responsible for the activity were identified using FTIR spectra, and the safety of the best synergistic blends was evaluated against HaCaT keratinocytes. The aim was to provide a ratio-guided, evidence-

based foundation for developing biocompatible plant-based topical antifungals that are effective against resistant dermatophytes.

2. MATERIALS AND METHODS

2.1 Chemicals and media

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

2.2 Collection and authentication of plant material

Five medicinal plants with reported antifungal properties were used: *Trigonella foenum-graecum* L. (RRCBI-mus105), *Piper betle* L. (RRCBI-mus113), *Curcuma longa* L. (RRCBI-mus277), *Piper tuberculatum* L. (RRCBI-mus454) and *Mangifera indica* L. (RRCBI-mus110). Leaves, rhizomes, and seeds of mature plants were gathered from around the Jnana Bharathi campus of Bangalore University and the Gandhi Krishi Vigyana Kendra (GKVK), Bengaluru. Dr. V. Ramarao, Research Officer at the Central Council for Research in Ayurvedic Sciences (CCRAS), Bengaluru 560109, authenticated every sample, and voucher specimens were stored for reference.

2.3 Preparation of extracts

Leaves, rhizomes, seeds, and pods were rinsed two or three times in tap water, shade-dried for 15 days, and ground to a powder. Each powdered sample was subjected to successive Soxhlet extraction in the chosen solvents. The extracts were concentrated, dried, and stored at 4 °C until use [9].

2.4 Preparation of polyherbal extract combinations

The dried extracts were weighed and mixed in set proportions to test their combined activity. Binary blends used ratios of 1:1 and 1:0.5 (w/w); ternary blends used 1:0.5:0.5 (w/w/w); and quaternary blends used 1:0.5:0.5:0.5 (w/w/w/w). To probe the effect of the ratio, further proportions were prepared: 0.5:0.5, 0.5:1, 1:0.5, and 1:1 for the binary sets, with matching permutations for the ternary and quaternary sets. Each mixture was vortex-homogenized, prepared fresh before analysis, dissolved in the appropriate solvent to the working concentration, and stored at 4 °C.

2.5 Antifungal assay (poisoned-food technique)

The activity against Trichophyton rubrum and Trichophyton mentagrophytes was measured using the poisoned food technique. Each combination was incorporated at a known concentration into molten Sabouraud's dextrose agar, poured into Petri plates, and inoculated at the center with a 5 mm mycelial disc from an actively growing culture. Ketoconazole plates served as positive controls, and extract-free plates served as negative controls. Plates were held at 28 ± 2 °C, after which the radial mycelial growth was measured, and the percentage inhibition was calculated against the untreated control. When the combined activity exceeded that of the individual extracts, synergy was inferred [10].

2.6 MIC and FIC index determination

The minimum inhibitory concentration (MIC) of the most active blends was determined using broth microdilution, following CLSI guidance for filamentous fungi [25]. The interaction type was quantified using the fractional inhibitory concentration (FIC) index, calculated as follows: FIC = (MIC of A in combination / MIC of A alone) + (MIC of B in combination / MIC of B alone). By convention, an FIC ≤ 0.5 was considered synergistic, 0.5 < FIC ≤ 1.0 as additive, 1.0 < FIC ≤ 4.0 as indifferent, and FIC > 4.0 as antagonistic [3].

2.7 Fourier-transform infrared (FTIR) spectroscopy

The functional groups in the synergistic blends were analyzed using FTIR spectroscopy on an instrument fitted with an attenuated total reflectance (ATR) accessory. Approximately 1–2 mg of each dried sample was placed on the ATR crystal, and spectra were recorded across 4000–400 cm^{-1} at 4 cm^{-1} resolution with 32 scans per sample. A background spectrum was obtained before each run, and the absorption bands were assigned to functional groups by comparison with standard correlation charts and published work [26].

2.8 In vitro cytotoxicity (MTT assay)

The cytotoxicity of the synergistic blends was tested on HaCaT human keratinocytes [27] using the MTT assay [28]. Cells were grown in DMEM with 10% fetal bovine serum and 1% antibiotic–antimycotic solution at 37 °C under 5% CO_2. They were seeded at 1×10^4 cells per well in 96-well plates, left to adhere overnight, and then exposed to graded concentrations (31.25–500 µg/mL) for 48 h, with doxorubicin (1 µM) as the positive control. MTT solution (20 µL, 5 mg/mL) was added and left for 3–4 h, the formazan crystals were dissolved in 100 µL DMSO, and the absorbance was measured at 570 nm. Cell viability (%) was calculated as (absorbance of treated / absorbance of control) × 100, and percentage inhibition was calculated as 100 − viability. IC_{50} values came from dose–response curves fitted in GraphPad Prism.

2.9 Statistical analysis

Each experiment was performed in triplicate, and the results are presented as the mean ± standard deviation (SD). Differences between treatments were tested using one-way ANOVA with Tukey's post-hoc test, considering $p < 0.05$ as significant.

3. RESULTS

3.1 Antifungal activity of binary (two-plant) combinations

Twenty-four two-plant blends were tested in four proportions against both the pathogens. Their activity varied widely, confirming that the choice of plants and the ratio between them both count [10]. For *T. rubrum*, TF+CL at 0.5:0.5 gave the deepest cut in mycelial growth (83.6 ± 1.5%), trailed by PB+CL (1:0.5; 83.3 ± 1.6%), PB+TF (0.5:1; 82.5 ± 2.8%) and PB+CL (0.5:0.5; 82.2 ± 2.2%). This aligns with existing knowledge regarding the curcuminoids of *C. longa* and the phenolics of *Trigonella foenum -graceum* and *Piper betel* [16,17,20]. T. mentagrophytes was the softer target overall. The strongest inhibitors were TF+PT (0.5:0.5; 97.7 ± 2.2%) and PB+PT (0.5:1; 97.4 ± 2.6%), with PB+TF (0.5:1; 93.6 ± 3.3%) close behind. The greater vulnerability of *T. mentagrophytes* is most likely due to differences in its cell wall composition and membrane permeability, which allow phytoconstituents to enter more readily [6]. Representative poisoned-food plates for the binary combinations are shown in Figures 2 and 3.

Table 1: Percentage inhibition of mycelial growth by the most active binary combinations (mean ± SD).

Combination	Proportion	*T. rubrum* (%)	*T. mentagrophytes* (%)
TF+CL	0.5:0.5	83.6 ± 1.5**	–
PB+CL	1:0.5	83.3 ± 1.6*	–
PB+TF	0.5:1	82.5 ± 2.8**	93.6 ± 3.3**
PB+CL	0.5:0.5	82.2 ± 2.2**	–
TF+MI	0.5:0.5	81.4 ± 3.7**	–
TF+PT	0.5:0.5	–	97.7 ± 2.2**
PB+PT	0.5:1	–	97.4 ± 2.6**
PB+PT	0.5:0.5	–	92.1 ± 3.9**
TF+PT	0.5:1	–	92.2 ± 0.6**

*p < 0.05; **p < 0.01 versus the untreated control. PB, *Piper betle*; TF, *Trigonella foenum-graecum*; CL, *Curcuma longa*; MI, *Mangifera indica*; PT, *Piper tuberculatum*.

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Figure 1. Antifungal activity of the most active binary combinations

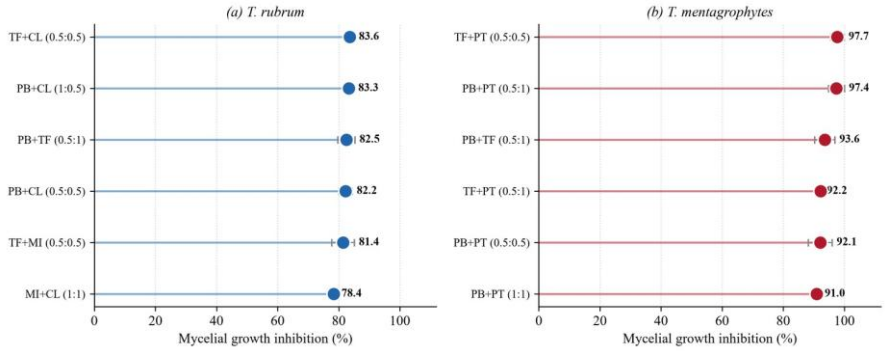


Figure 1: Percentage inhibition of mycelial growth by the most active binary combinations against (a) T. rubrum and (b) T. mentagrophytes. Values are expressed as mean ± SD (n = 3).

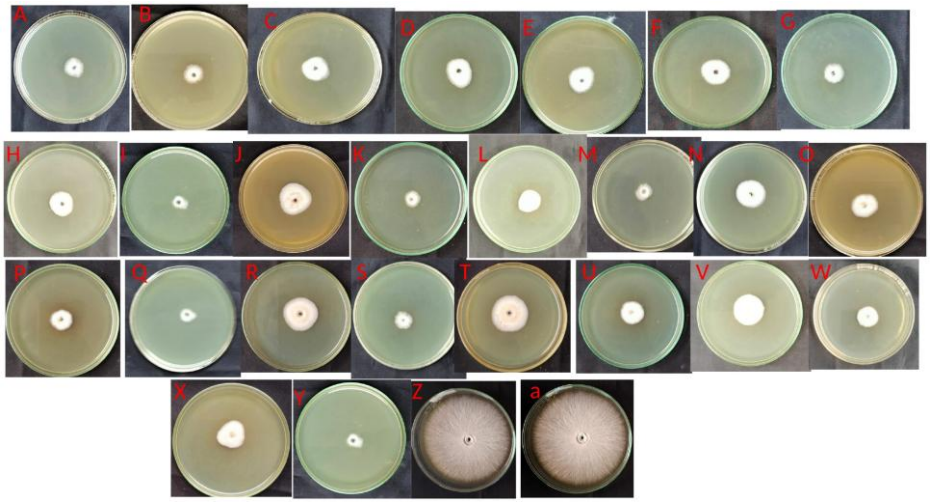


Figure 2: Antifungal activity of binary (two-plant) combinations against T. rubrum in the poisoned-food assay. A–D, PB+TF; E–H, PB+CL; I–L, PB+MI; M–P, TF+CL; Q–T, TF+MI; U–X, MI+CL (each at 0.5:0.5, 0.5:1, 1:0.5 and 1:1); Y, positive control (ketoconazole); Z, vehicle control; a, organism control.

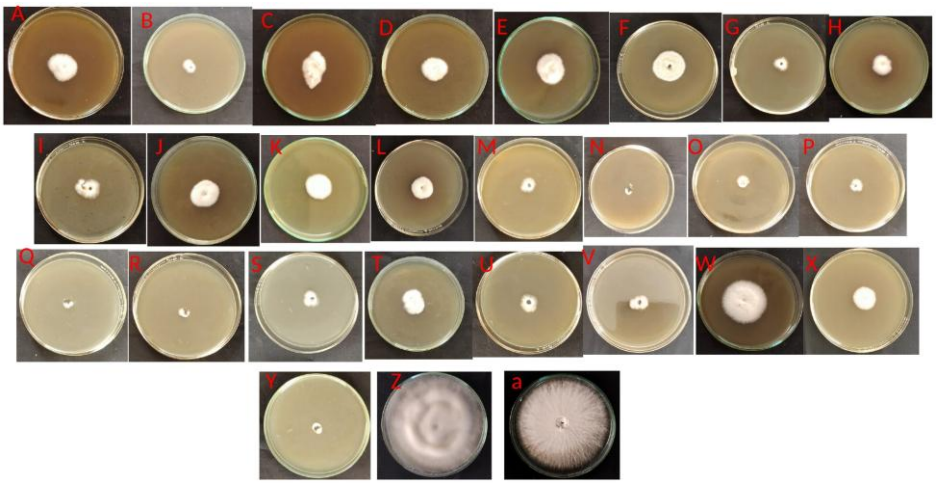


Figure 3: Antifungal activity of binary (two-plant) combinations against T. mentagrophytes in the poisoned-food assay. A–D, PB+TF; E–H, PB+PT; I–L, PB+MI; M–P, TF+PT; Q–T, TF+MI; U–X, MI+PT (each at 0.5:0.5, 0.5:1, 1:0.5 and 1:1); Y, positive control (ketoconazole); a, vehicle control; Z, organism control.

The ratio between the two extracts shifted the activity in a significant manner. For most pairs, the 0.5:0.5 and 0.5:1 blends surpassed the even 1:1 blend, indicating that excessive use of either extract can be detrimental, and a balanced or partner-weighted mix is optimal. PB+TF, for instance, increased from 70.1% at 0.5:0.5 to 82.5% at 0.5:1 against *T. rubrum*, while PB+PT peaked against *T. mentagrophytes* at 0.5:1 (97.4%). This type of ratio dependence is the fingerprint of a genuine interaction rather than a plain dose addition [10].

3.2 Antifungal activity of ternary (three-plant) combinations

Thirty-two three-plant blends were screened to determine whether a third plant was beneficial. This was done for only one pathogen. Against *T. rubrum*, PB+TF+MI at 1:0.5:0.5 showed the highest inhibition in the entire study (87.1 ± 0.5%), edging past every binary blend, and the same trio at 1:1:0.5 was almost as potent (86.8 ± 2.5%). Mango leaf flavonoids plausibly exert much of this activity, in line with earlier reports [22,23]. Against *T. mentagrophytes* the three-plant blends fell away sharply, the best being only 51.9 ± 0.5% for PB+MI+PT (0.5:1:0.5). A third extract can dilute the most potent constituents or set off antagonism; therefore, the ratio matters more than the sheer count of components [15]. Representative plates are shown in Figures 4 and 5.

Table 2: Most active ternary combinations against each dermatophyte (mean ± SD).

Organism	Combination	Proportion	Inhibition (%)
T. rubrum	PB+TF+MI	1:0.5:0.5	87.1 ± 0.5**
T. rubrum	PB+TF+MI	1:1:0.5	86.8 ± 2.5**
T. rubrum	PB+MI+CL	1:0.5:1	79.1 ± 0.9**
T. mentagrophytes	PB+MI+PT	0.5:1:0.5	51.9 ± 0.5*
T. mentagrophytes	PB+MI+PT	0.5:1:1	49.5 ± 0.5*
T. mentagrophytes	TF+PT+MI	1:1:0.5	48.8 ± 2.7*

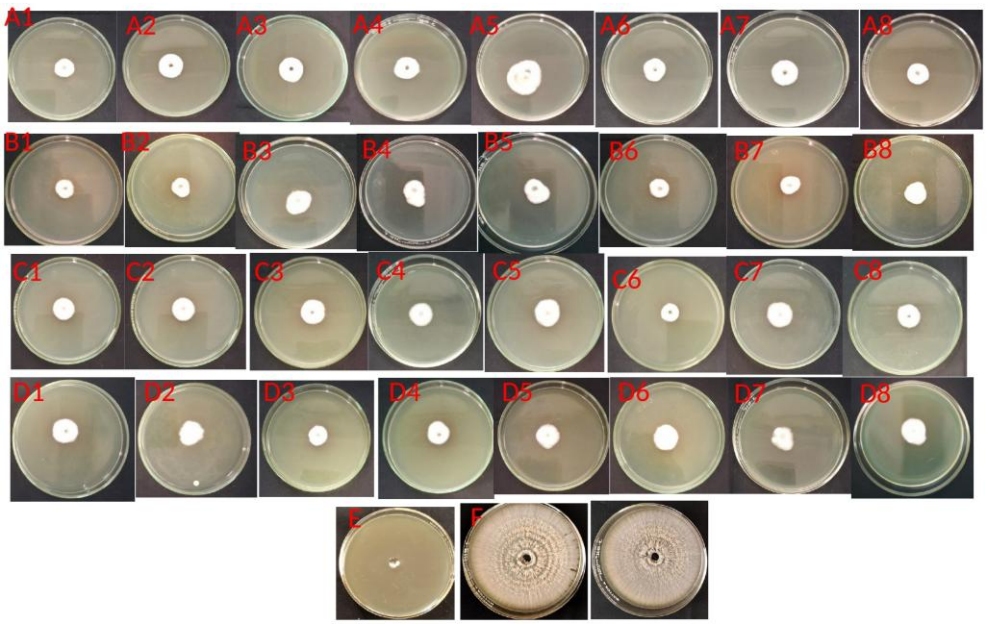


Figure 4: Antifungal activity of ternary (three-plant) combinations against T. rubrum. A1–A8, PB+TF+CL; B1–B8, PB+TF+MI; C1–C8, PB+MI+CL; D1–D8, TF+CL+MI (each at 0.5:0.5:0.5, 0.5:0.5:1, 0.5:1:1, 0.5:1:0.5, 1:0.5:0.5, 1:0.5:1, 1:1:0.5 and 1:1:1); E, positive control (ketoconazole); F, vehicle control; G, organism control.

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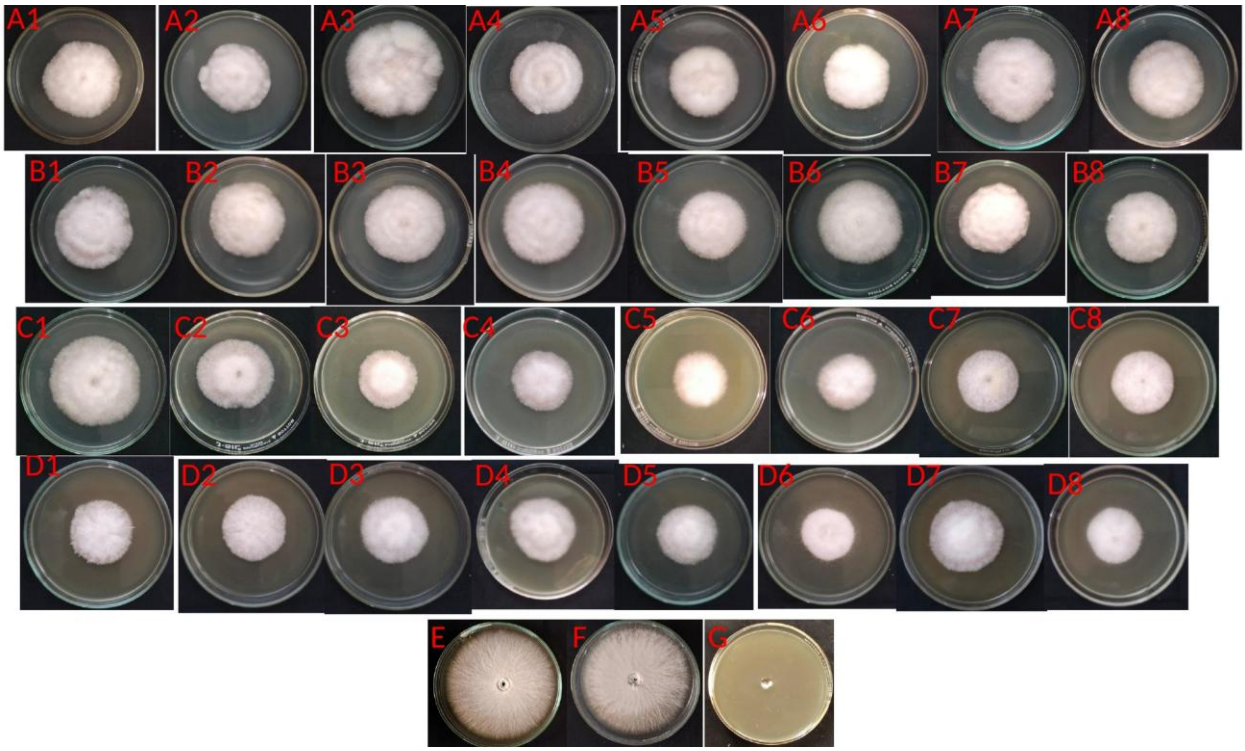


Figure 5: Antifungal activity of ternary (three-plant) combinations against T. mentagrophytes. A1–A8, PB+TF+PT; B1–B8, PB+TF+MI; C1–C8, PB+MI+PT; D1–D8, TF+PT+MI (each at 0.5:0.5:0.5, 0.5:0.5:1, 0.5:1:1, 0.5:1:0.5, 1:0.5:0.5, 1:0.5:1, 1:1:0.5 and 1:1:1); G, positive control (ketoconazole); F, vehicle control; E, organism control.

3.3 Antifungal activity of quaternary (four-plant) combinations

Fourteen four-plant blends were created to test whether stacking four plants would increase activity. It did not. Every blend worked to some degree, but inhibition generally was below that of the best two- and three-plant mixtures. The strongest result against *T. rubrum* was obtained with PB+CL+TF+MI (1:1:0.5:1) at 61.0 ± 2.8%, and against *T. mentagrophytes* with PB+PT+TF+MI (1:0.5:0.5:1) at 64.3 ± 1.8%. This drop-off is what one would expect from phytochemical crowding, where too many bioactive molecules compete for the same targets, cut into each other's bioavailability, or simply get in each other's way [10,12]. Representative plates are shown in Figures 6 and 7.

Table 3: Most active quaternary combinations against each dermatophyte (mean ± SD).

Organism	Combination	Proportion	Inhibition (%)
T. rubrum	PB+CL+TF+MI	1:1:0.5:1	61.0 ± 2.8**
T. rubrum	PB+CL+TF+MI	0.5:0.5:0.5:1	59.4 ± 2.7**
T. rubrum	PB+CL+TF+MI	0.5:1:1:1	57.7 ± 6.3**
T. mentagrophytes	PB+PT+TF+MI	1:0.5:0.5:1	64.3 ± 1.8**
T. mentagrophytes	PB+PT+TF+MI	1:1:1:1	60.7 ± 4.6**
T. mentagrophytes	PB+PT+TF+MI	0.5:0.5:1:0.5	58.1 ± 3.3*

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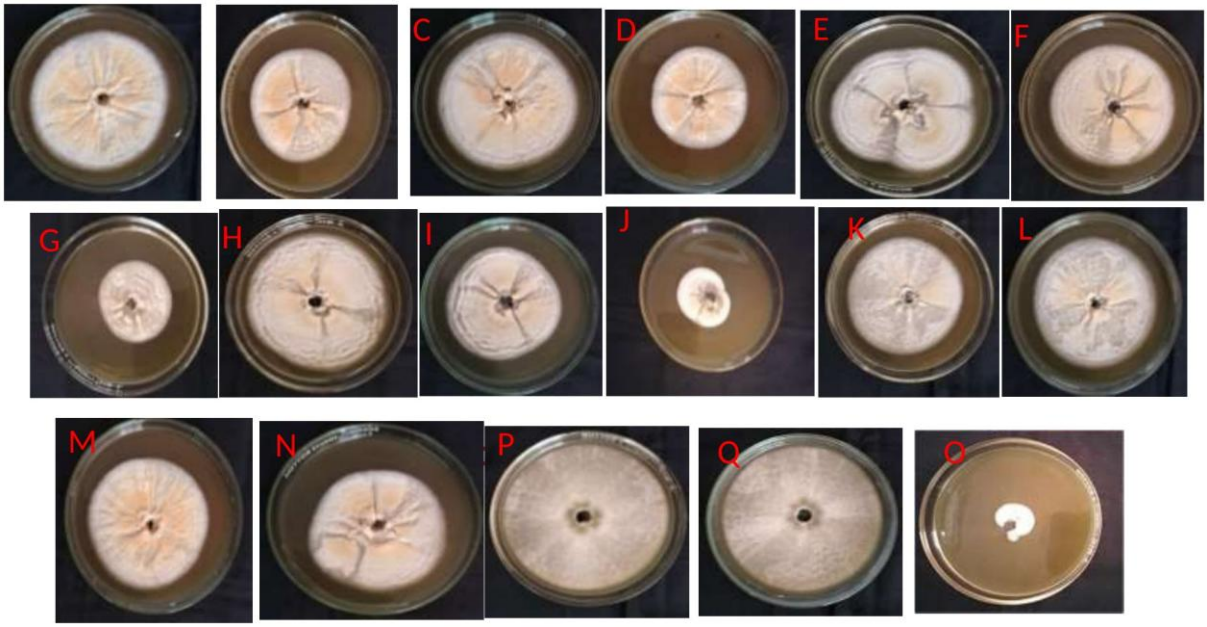


Figure 6: Antifungal activity of quaternary (four-plant) combinations against T. rubrum. A–N, PB+CL+TF+MI at different proportions; O, positive control (ketoconazole, 25 µg/mL); P, vehicle control; Q, organism control.

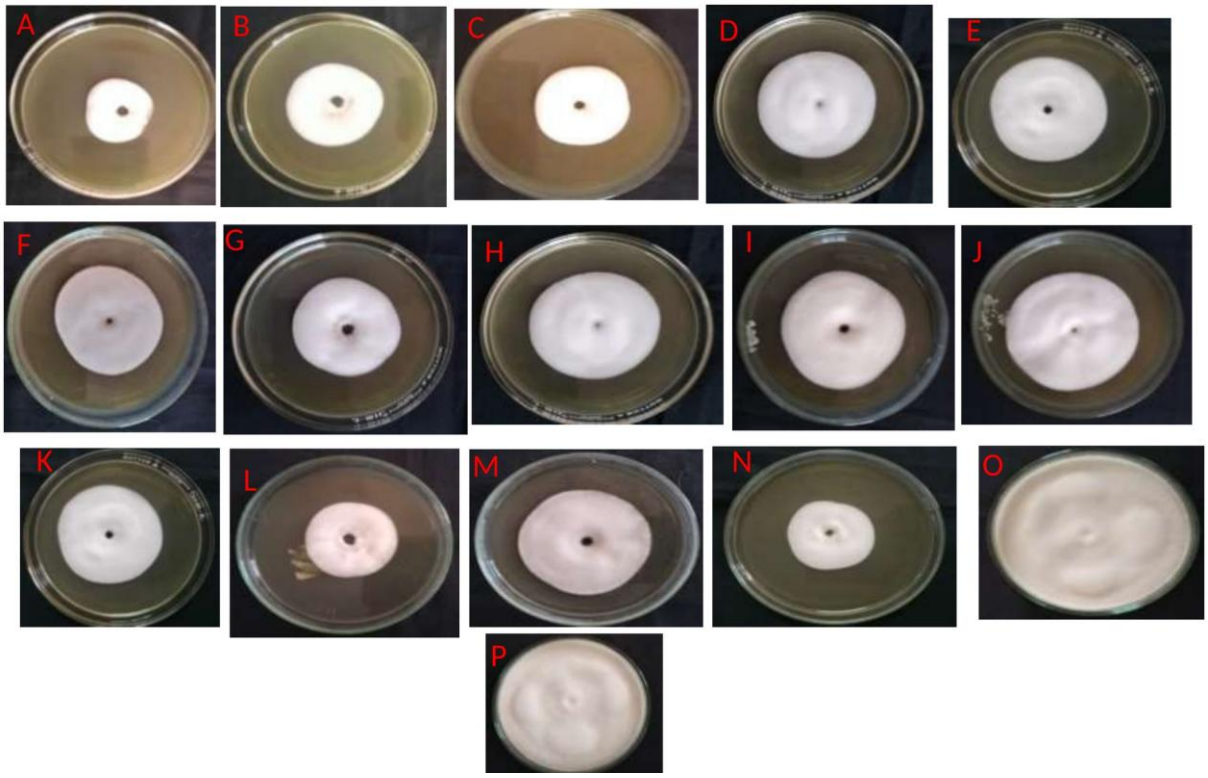


Figure 7: Antifungal activity of quaternary (four-plant) combinations against T. mentagrophytes. A–N, PB+PT+TF+MI at different proportions; O, organism control; P, vehicle control.

Figure 8. Peak antifungal activity across combination complexity

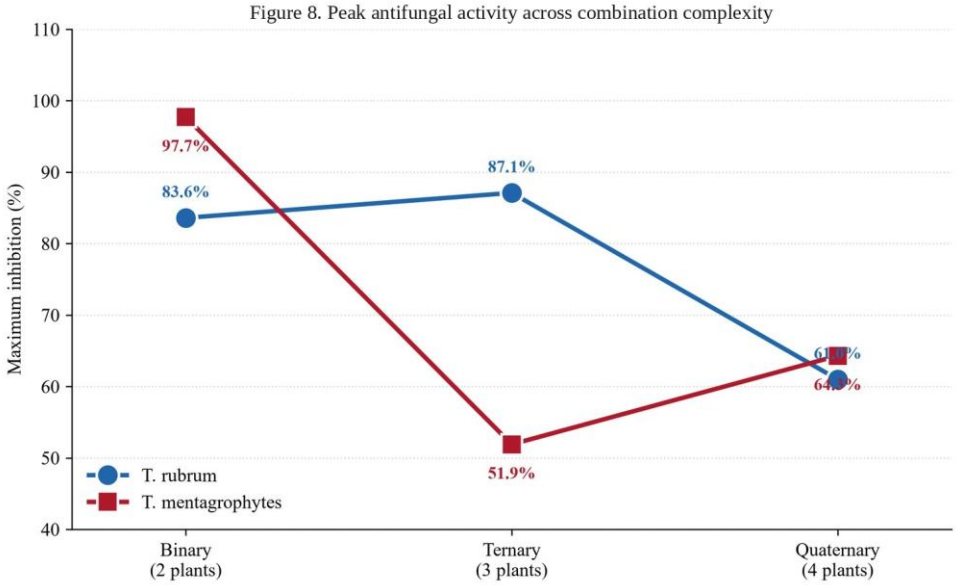


Figure 8: Maximum inhibition of mycelial growth was achieved by binary, ternary, and quaternary combinations against each dermatophyte, showing that activity does not scale with the number of plant components.

Figure 8 shows the central trend of screening. For *T. rubrum*, the best result increased only slightly from binary (83.6%) to ternary (87.1%) and then decreased significantly in the quaternary stage (61.0%). For *T. mentagrophytes*, the fall was steeper and steady, from 97.7% to 51.9% and 64.3%. Read together, these numbers make one point plainly: adding more plant extracts does not buy more activity, and past a certain point it costs you [12,15].

3.4 MIC and FIC index – nature of the interactions

Out of 70 blends screened against *T. rubrum*, the 12 most active were subjected to MIC and FIC testing. Three combinations were synergistic (FIC ≤ 0.5), six were additive, and three were indifferent, with no antagonistic combinations. The sharpest synergy belonged to PB+CL (0.5:0.5; FIC 0.3) and the four-plant PB+CL+TF+MI at 0.5:1:1:1 and 1:1:1:0.5, both scoring FIC 0.4. For *T. mentagrophytes*, 11 blends were tested: one synergistic (PB+TF, 0.5:0.5; FIC 0.3), three additive, and seven indifferent, again with no antagonism. These patterns bear out the central claim: pick the right partners and you can lower the effective dose and reach real synergy [3,15].

Table 4: MIC and FIC indices of representative synergistic and additive combinations.

Organism	Combination	Proportion	MIC (mg/mL)	FIC	Interaction
T. rubrum	PB+CL	0.5:0.5	2.5	0.3	Synergistic
T. rubrum	PB+CL+TF+MI	0.5:1:1:1	5	0.4	Synergistic
T. rubrum	PB+CL+TF+MI	1:1:1:0.5	5	0.4	Synergistic
T. rubrum	TF+CL	0.5:0.5	1.25	0.7	Additive
T. mentagrophytes	PB+TF	0.5:0.5	1.25	0.3	Synergistic
T. mentagrophytes	TF+PT	0.5:0.5	2.5	0.5	Additive
T. mentagrophytes	PB+PT+TF+MI	1:0.5:0.5:1	2.5	0.7	Additive

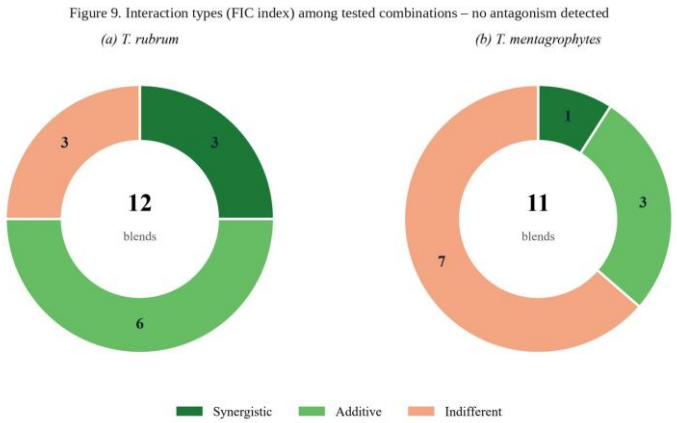


Figure 9: Distribution of interaction types, classified by the FIC index, among the combinations selected for MIC testing against each dermatophyte. No antagonistic interactions were detected.

Figure 9 shows that synergy was the exception, not the rule. Additive and indifferent interactions dominated, and for *T. mentagrophytes*, seven of the eleven tested blends were merely indifferent. What matters just as much is the negative results. Across all 23 combinations tested for either pathogen, none showed antagonism, indicating that these extracts can be co-formulated without one undercutting another, even when synergy fails to appear [3].

3.5 FTIR analysis

FTIR spectroscopy linked the antifungal activity to specific chemistry. Both the *Piper betle* + *Curcuma longa* blend and the four-plant PB+CL+TF+MI blend showed a broad band at 3400–3300 cm^{-1} (O–H/N–H stretching of phenols, flavonoids, curcuminoids, and proteins), bands at 2925–2850 cm^{-1} (aliphatic C–H stretching of terpenoids and alkanes), a signal at 1700–1600 cm^{-1} (C=O/C=C stretching of carbonyl and aromatic compounds), and a peak at 1545–1505 cm^{-1} (aromatic C=C / N–H bending). Further bands at 1455–1400 and 1270–1020 cm^{-1} (CH_2/CH_3 bending, C–O and C–O–C stretching), plus features below 1000 cm^{-1}, are characteristic of alcohols, ethers, glycosides, and substituted aromatic rings. Groups of this kind are typical of antifungal phenolics, flavonoids, and terpenoids [9,26], and their survival in the blended spectra indicates that the extracts remain chemically compatible once mixed. The spectra of the individual extracts are shown in Figure 10.

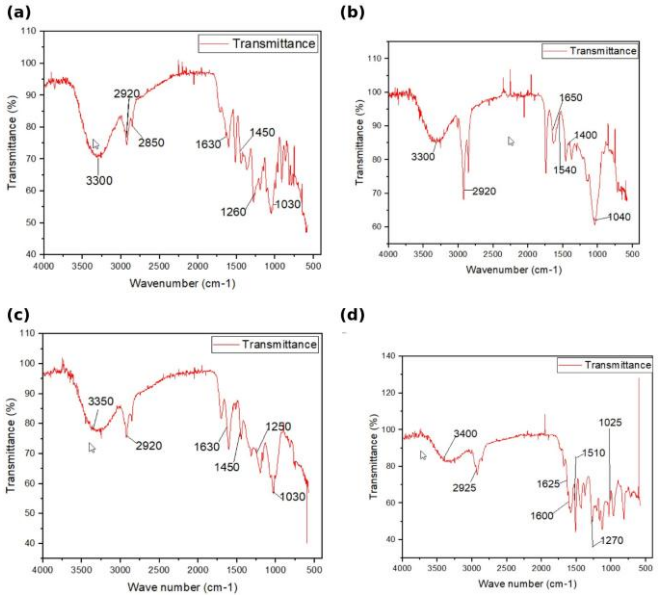


Figure 10: FTIR spectra of the individual plant extracts: (a) ethanolic extract of Piper betle, (b) methanolic extract of Trigonella foenum-graecum, (c) methanolic extract of Mangifera indica and (d) ethanolic extract of Curcuma longa.

3.6 Cytotoxicity (MTT assay on HaCaT cells)

The two lead blends were then checked against HaCaT keratinocytes for 48 h [27,28]. The Piper betle + Curcuma longa blend (Sy-1b) steadily lost viability with increasing dose, from 61.95 ± 0.02% at 31.25 µg/mL to 27.21 ± 0.02% at 500 µg/mL (inhibition 38.05–72.79%), which set its IC_{50} at 91.53 µg/mL, indicating moderate toxicity. The four-plant blend (Sy-2b, PB+CL+TF+MI) was gentler on the cells, with viability slipping from 65.27 ± 0.01% to 37.34 ± 0.005% across the same range (inhibition 34.73–62.66%) for an IC_{50} of 229.81 µg/mL, in the low-toxicity band. Doxorubicin, the positive control at 1 µM, wiped out about 96% of the cells. Therefore, both blends kept keratinocytes reasonably intact at doses that suppressed fungi, indicating selective action and a workable margin for topical use [29]. Cell morphology after 48 h of exposure is shown in Figure 11.

Table 5: Cytotoxicity of the synergistic blends against HaCaT keratinocytes (48 h).

Sample	Composition	Viability at 31.25–500 µg/mL (%)	IC_{50} (µg/mL)	Classification
Sy-1b	PB+CL	61.95 → 27.21	91.53	Moderate toxicity
Sy-2b	PB+CL+TF+MI	65.27 → 37.34	229.81	Low toxicity
Ketoconazole	Positive control (1 µM)	3.75–3.87	–	Reference cytotoxic

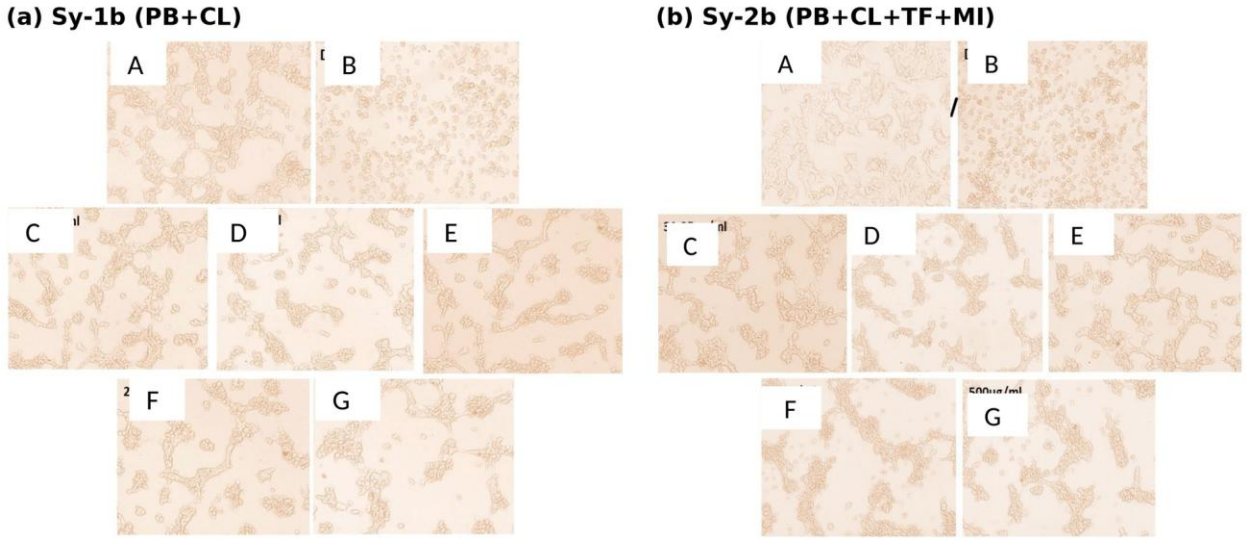


Figure 11: Morphology of HaCaT keratinocytes after 48 h of exposure to (a) Sy-1b (PB+CL) and (b) Sy-2b (PB+CL+TF+MI). In each panel: A, untreated control; B, positive control (doxorubicin); C–G, 31.25, 62.5, 125, 250 and 500 µg/mL, respectively. Images were captured at 10× objective magnification (100× total magnification).

Figure 12. Concentration-dependent cytotoxicity on HaCaT cells (48 h)

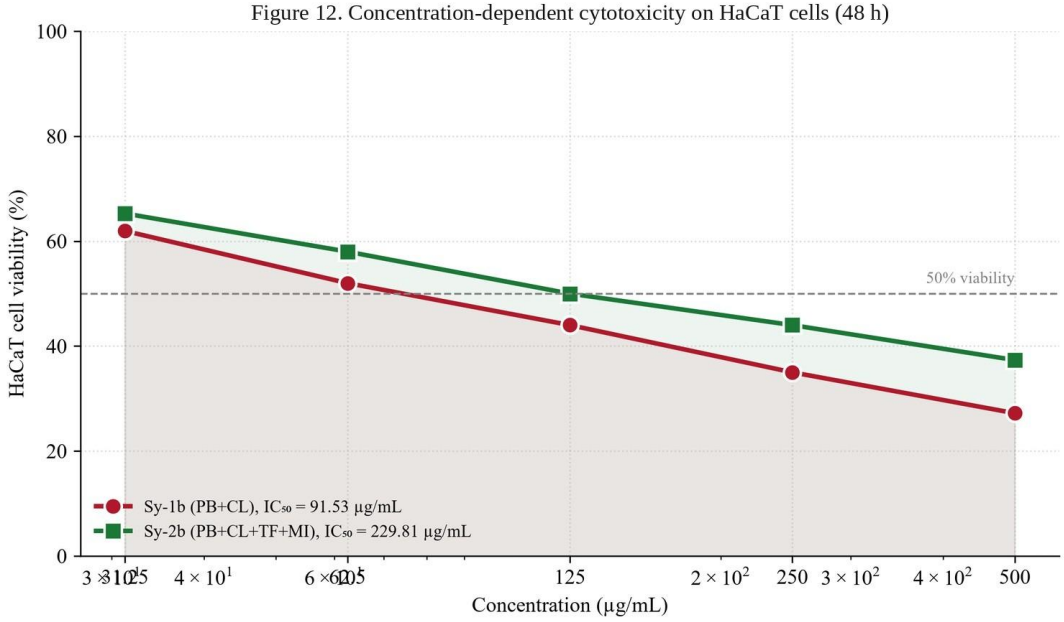


Figure 12: The two synergistic blends exhibited a concentration-dependent decline in HaCaT keratinocyte viability over the range of 31.25–500 µg/mL (48 h exposure). The dashed line indicates 50% viability.

One contrast in Table 5 deserves further discussion. The four-plant blend was the weaker antifungal, yet its IC_{50} was nearly two-and-a-half times higher than that of the two-plant blend, so it caused far less damage to keratinocytes. In other words, potency and toxicity moved together, and the milder formulation resulted in a comparable or better therapeutic window. This trade-off is worth weighing carefully when a lead formulation is selected for topical development [29].

4. DISCUSSION

The headline finding is easy to state as follows. Combining plant extracts can significantly enhance antifungal activity against dermatophytes; however, the effectiveness depends on the choice of partners and their ratio, rather than the number of plants used [10,15]. Every top-ranking blend — TF+CL (0.5:0.5) and PB+TF+MI (1:0.5:0.5) against *T. rubrum*, TF+PT, and PB+PT against *T. mentagrophytes* — used only two or three well-matched extracts. Their potency is best understood as several mechanisms working in concert: the phenolics, flavonoids, and curcuminoids of *Curcuma longa* and *Trigonella foenum-graecum* alongside the amides and terpenoids of the *Piper* species, together disrupting membranes, blocking ergosterol synthesis, driving oxidative stress, and interfering with fungal enzymes [11,17,18,24].

T. mentagrophytes was the easier organism to hit with the binary blends, several of which showed 92% inhibition. The most likely reason for this is the cell wall and membrane architecture, which allows the active phytoconstituents to cross more freely [6,30]. This ranking was reversed for the three- and four-plant blends, where *T. rubrum* was the more susceptible of the two. The reversal itself is informative. This indicates that synergy is tied to particular targets and ratios rather than being a fixed trait of any one extract mixture [3].

The clearest lesson comes from the ternary and quaternary blends, whose activity often sagged. The addition of extra phytochemicals did not simply add up. Instead, they seem to have competed for shared targets, thinned out the most potent constituents, and occasionally worked against each other, dragging the net effect down. This crowding effect has been observed in other complex herbal systems [10,12], and it argues for a design rule that is almost counterintuitive: fine-tune a few good partners rather than chase chemical diversity for its own sake.

The MIC and FIC data provide the argument numbers. True synergy, at FIC ≤ 0.5, was observed in only a few blends, with PB+CL against *T. rubrum* and PB+TF against *T. mentagrophytes* being the most prominent, while most active combinations were in the additive or indifferent range and none were antagonistic [3]. For anyone developing a formulation, the clean absence of antagonism is reassuring, as it means that these extracts can share a product without cancelling each other out. The synergistic blends further lowered the effective inhibitory dose, which could reduce drug requirements and slow the emergence of resistance [7,8].

FTIR confirmed all of this at the chemical level. Both synergistic blends carried the hydroxyl, aliphatic C–H, carbonyl, aromatic C=C, and C–O signatures of phenolics, flavonoids, terpenoids, and glycosides [9,26]. Their persistence in the combined spectra shows that the constituents remain compatible and do not visibly break down upon mixing, which is important for a topical product that has to remain stable on a shelf.

The cytotoxicity results support the case for these leads. Both synergistic blends left HaCaT cells largely intact at the doses that suppressed the fungi, and the four-plant blend, in particular, with an IC_{50} of 229.81 µg/mL, barely troubled the keratinocytes [28,29]. Hitting the dermatophyte while sparing the host's skin is exactly the profile a topical antifungal is meant to have [31].

Set against the wider literature, these results both agree with and extend previous work. Yang *et al.* [13] found that traditional Chinese medicines hit superficial fungi harder in combination than alone, and Tiwana *et al.* [14] showed that specific triphala ratios boosted antibacterial activity in ways that single extracts never predicted. The present study makes a parallel case for dermatophytes and adds a practically useful twist: the link between chemical complexity and efficacy is not a straight line but a downward curve. The proposed mechanisms — curcumin-driven ROS generation and azole potentiation [18,19], phenol-led membrane disruption from betel and mango constituents [16,22], and terpenoid- and amide-mediated permeabilization from the Piper species and fenugreek [11,20,24] — provide a coherent molecular reading of both the synergy and species reversal.

However, some limitations should be considered. The screening was performed in vitro against two dermatophyte species using the poisoned-food and broth-microdilution methods; therefore, activity against clinical isolates, resistant strains, and other genera, such as Microsporum and Epidermophyton, still needs to be confirmed. The FIC index, although useful, describes pairwise interactions and only approximates what happens in three- and four-component mixtures; therefore, synergy calls warrant caution [3,15]. Cytotoxicity was assessed using a single keratinocyte line at one exposure time, and the active phytochemicals were analyzed only at the functional group level rather than being isolated individually. Future work should therefore include chromatographic identification of the actives, time-kill and ergosterol-binding assays to determine the mechanism, ex vivo skin-permeation studies, and in vivo efficacy and safety testing of the lead blend in an animal model of dermatophytosis.

5. CONCLUSION

Carefully chosen combinations of *Piper betle*, *Trigonella foenum-graecum*, *Curcuma longa*, *Mangifera indica*, and *Piper tuberculatum* act strongly and in a ratio-dependent manner against Trichophyton rubrum and Trichophyton mentagrophytes. Two- and three-plant blends — TF+CL, PB+TF+MI, TF+PT, and PB+PT—outperformed the busier four-plant mixtures, and FIC analysis confirmed genuine synergy in select pairs with no antagonism. FTIR linked the activity to phenolic, flavonoid, terpenoid, and glycosidic groups, while the HaCaT results showed a safety margin wide enough to matter. Taken together, this study offers a rational, ratio-guided route to biocompatible plant-based topical antifungals and makes a solid case for the use of synergistic blends in in vivo and mechanistic studies.

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AUTHOR CONTRIBUTIONS

Sushma H. N: Conceptualization, methodology, investigation, experimental analysis, data interpretation, and writing of the original manuscript, Investigation, experimental assistance, data collection Mrinalini devi U.G : manuscript review and data interpretation. Dr. T. Sharmila: Conceptualization, supervision, validation, critical review, and editing of the manuscript. All authors have read and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no known financial or personal conflicts of interest that could have influenced the research work presented in this manuscript.

ETHICS STATEMENT

This study involved in-vitro experimental investigations and did not include experiments involving human participants or animals. Therefore, approval from an Institutional Ethics Committee was not required.

DATA AVAILABILITY STATEMENT

The data generated and analyzed during the present study are included in the manuscript. Additional supporting data are available from the corresponding author upon reasonable request.

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